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The role and the prevalence of *icaABDC*, *aap* and *bhp* genes in the virulence of *Staphylococcus epidermidis* Portuguese isolates

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"Scientists wonder how certain things work, so they try more and more to find out how and why. Whether or not their work will lead to something useful, they don't care, because they don't know, and for that matter, they're not that interested. If you develop science only with the idea to do something useful, then your chances of discovering something useful are less than if you apply your mind to finding something essential."

-Gerhard Herzberg

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SCIENTIFIC OUTPUTS

Under the terms of the n.º 1 do artigo 34.º do Decreto-Lei n.º 74/2006, publicado em Diário da República, 1.ª série, n.º 60 de 24 de Março de 2006, e republicado pelo Decreto-Lei n.º 115/2013, publicado em Diário da República, 1.ª série, n.º 151 de 7 de Agosto de 2013, que procede à terceira alteração ao Decreto-Lei n.º 74/2006, de 24 de Março de 2006, the author hereby declared that has actively participated in the design and technical execution of the work, interpretation of the results and manuscript preparation of the original articles included in this thesis. Under the terms of the referred Decreto-Lei, the author hereby declared that the following original articles/communications were prepared in the scope of this thesis.

PAPERS IN PEER REVIEWED JOURNALS

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BOOK CHAPTER

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ORAL COMMUNICATION

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POSTER COMMUNICATION

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3D	Tridimensional
aa	amino acid
Aap	Accumulation-associated protein
ATCC	American Type Culture Collection
Agr	Acessory gene regulator
AI-2	Autoinducer-2
AIP	Autoinducing peptides
AtlE	Autolysin E
BCA	Bicinchoninic acid
Bhp	biofilm-homolog *S. aureus* protein
bp	base pair
BSIs	Bloodstream infections
$CaCl_2$	Calcium chloride
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CFU	Colony-forming unit(s)
CAUTIs	Catheter-associated urinary tract infections
CLABSIs	Central line-associated bloodstream infections
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
CoNS	Coagulase-negative staphylococci
CV	Crystal violet
CVC	Central venous catheter
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
eDNA	Extracellular DNA
ECDC	European Centre for Disease Prevention and Control
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Embp	Extracellular matrix binding protein
EPS	Extracellular polymeric substance
EU	European Union
Fbe/SdrG	Fibrinogen-binding protein
FITC	Fluorescein isothiocyanate
FW	Forward
gDNA	genomic DNA
HAIs	Healthcare-associated infections
Ica	Intercellular cluster adhesin
MALDI-TOF	Matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry

MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSE	Methicillin-susceptible *Staphylococcus epidermidis*
n	Number
NaCl	Sodium chloride
$NaIO_4$	Sodium meta-periodate
NCCLS	National committee for clinical laboratory standards
PBP2a	Penicillin-binding protein 2
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-*N*-acetylglucosamine
PSMs	Phenol soluble modulins
PI	Propidium iodide
QS	Quorum-sensing
OD	Optical density
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RIP	RNAIII-inhibiting peptide
RNA	Ribonuclecleic acid
rpoB	RNA polymerase β subunit gene
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RV	Reverse
SCC	Staphylococcal cassette chromosome
SD	Standard deviation
SSIs	Surgical site infections
Ssp	Staphylococcal surface-associated protein
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UTIs	Urinary tract infections
USA	United States of America
VAPs	Ventilator-associated pneumonias
WGA	Wheat germ agglutinin

ABSTRACT

Staphylococcus epidermidis is the most common causative agent of relapsing and persistent hospital-acquired infections associated with indwelling medical devices. Since this bacterium belongs to the human skin microbiota, auto- and/or cross-infections are highly frequent. Moreover, the increased use of medical devices and the great ability of *S. epidermidis* to colonize them and form biofilms has therefore raised this otherwise innocuous skin commensal bacterium to a status of major opportunistic pathogen. Biofilms are complex three-dimensional structures of bacteria encased in a self-protecting matrix. This structure efficiently protects bacteria within the biofilm from the host immune system and antimicrobial killing, threatening the achievements of modern medicine. In fact, resistance to conventional antimicrobials, mainly multidrug resistance, is rising due to their increased and sometimes improper use of antibiotics in both healthcare and community settings, therefore impairing the patients' treatment. In *S. epidermidis*, *icaADBC*–dependent and –independent (proteinaceous) mechanisms can be considered in biofilm development. The one dependent on *icaADBC* is the best described and relies on the synthesis of polysaccharide intercellular adhesin (PIA) encoded by the *icaADCB* operon. Aside from PIA, the accumulation-associated protein (Aap) and the biofilm-associated protein (Bap) of *S. aureus* protein (Bhp), represent some of the best-characterized biofilm determinants involved in protein-mediated biofilm mechanism.

This thesis starts with the description of distinct optimizations of two important techniques for biofilm analysis: biofilm quantification and quantification of biofilm gene expression. Furthermore, the antimicrobial susceptibilities of clinical *S. epidermidis* isolates collected in a tertiary-care hospital (Hospital de Santo António) were described and their carriage of *icaA*, *aap* and *bhp* biofilm-mediating genes and subsequent biofilm formation was characterized. The first findings revealed very high antimicrobial resistance among *S. epidermidis* isolates, mainly multidrug resistance, in this Portuguese hospital. An association was found between *mecA* and multidrug resistance phenotype. In addition, by analyzing the expression of these biofilm-mediating genes, it was observed that *icaA* is predominantly associated with *S. epidermidis* biofilm development. It was also

confirmed that *aap* is essential in proteinaceous biofilm development, although only *S. epidermidis* harboring the *icaA* gene had demonstrated the ability to develop thicker biofilms with a more complex biofilm organization. Importantly, the role of *bhp* in the biofilm formation remains uncertain. These findings were also confirmed by using commensal isolates. Overall, the findings described in this thesis allowed to better characterize the interaction and contribution of *icaA*, *aap* and *bhp* to the *S. epidermidis* biofilm formation process, and contributed to increase the knowledge on *S. epidermidis* device-related infections occurring in Portugal.

SUMÁRIO

Staphylococcus epidermidis é o mais frequente agente causador de infeções hospitalares, recorrentes e persistentes, associadas a dispositivos médicos implantados. Sendo esta bactéria parte integrante do microbioma da pele humana, auto-infeções e/ou infeções cruzadas são bastante frequentes. A utilização crescente de dispositivos médicos, associada à excelente capacidade de *S. epidermidis* em os colonizar e formar biofilmes, elevou o estatuto desta outrora inócua bactéria, ao de patogénio oportunista. Biofilmes são estruturas tridimensionais complexas de bactérias envoltas por uma matriz protetora. Esta estrutura protege as bactérias do sistema imunológico do hospedeiro assim como da atuação de antibióticos. A resistência aos antibióticos, principalmente a multirresistência, tem aumentado devido ao elevado uso, por vezes inapropriado, de antibióticos, quer nas unidades prestadoras de cuidados de saúde quer na comunidade, complicando assim o tratamento dos doentes. Na espécie *S. epidermidis* são considerados dois tipos de mecanismos que regulam o desenvolvimento de biofilmes: dependentes e independentes do operão *icaADBC*. Os dependentes de *icaADBC* são os mais estudados e dependem da síntese da adesina intracelular polissacarídica (PIA, em inglês), codificado pelo operão *icaADBC*. Os mecanismos independentes de PIA estão fortemente associados à produção da proteína associada à acumulação (Aap, do inglês *accumulation associated proteín*) e da proteína homóloga a Bap de biofilmes de *S. aureus* (Bhp, do inglês *biofilm-associated protein (Bap) of S. aureus protein*).

O trabalho incluído nesta tese inicia com a descrição da otimização efetuada a duas técnicas amplamente usadas na análise de biofilmes – as quantificações do biofilme e da expressão de genes envolvidos na formação do mesmo –,aplicadas também à análise de biofilmes de *S. epidermidis*. De seguida, foi descrita a suscetibilidade de vários isolados clínicos de *S. epidermidis,* provenientes de doentes tratados num hospital terciário (Hospital de Santo António - Centro Hospitalar do Porto), aos antibióticos mais comummente usados contra este tipo de infeções, assim como determinada a quantidade de biofilme formado por cada isolado, procedida pela caracterização genotípica. Estes resultados revelaram uma elevada taxa de resistência aos antimicrobianos principalmente

multirresistência tendo-se verificado uma associação entre o fenótipo da multirresistência e a presença do gene *mecA*. Posteriormente descreveu-se a relação entre a expressão dos genes associados à formação de biofilme e o respetivo crescimento do biofilme, tendo-se verificado que o gene *icaA* está diretamente e de forma predominante, ligado ao desenvolvimento do biofilme na espécie *S. epidermidis*. Por outro lado, verificou-se que o gene *aap*, mas não *bhp*, desempenha um papel relevante no desenvolvimento do biofilme aquando a ausência de *icaA*. Todavia, apenas isolados que possuam *ica* no seu genoma demostraram capacidade para formar biofilmes mais densos e estruturalmente mais complexos. Os resultados supramencionados foram consolidados através da inclusão de isolados da comunidade neste estudo. De uma forma geral, os resultados descritos nesta tese permitem melhor determinar a interação entre os genes *icaA*, *aap* e *bhp* e a contribuição individual destes no processo de formação de biofilme assim como contribuem para um entendimento mais alargado das infeções associadas a biofilmes de *S. epidermidis*, em Portugal.

CHAPTER 1

LITERATURE REVIEW

This chapter provides the state of the art knowledge on clinical and molecular aspects of the pathogenesis of *Staphylococcus epidermidis* infections related to implanted medical devices. Special emphasis was given to some of the most important biofilm-mediating genes and their contribution to biofilm development. In the last section of this chapter the main objectives of this thesis are described.

1.1. Clinical relevance of Staphylococci associated infections

The progress of the modern medicine due to enhanced medical knowledge and treatments led to an extensive use of indwelling medical devices such as central venous catheters, prosthetic joints, cardiac pacemakers and mechanical heart valves, vascular grafts, artificial lenses, cerebrospinal fluid shunts, amongst others [1]. Healthcare-associated infections (HAIs) or nosocomial infections, are defined as infections occurring during a stay in healthcare facilities that were neither present nor incubating at the time of hospital admission and only appear in patients hospitalized for 48 hours or longer [2]. These infections can take a chronic persistent course indicating that host innate and acquired immunity do not deal effectively with the causative organism(s), compromising the patient's quality of life [3]. Besides medical complications, these infections increase both morbidity and mortality, primarily among immunocompromised and critically ill patients, and also elderly people [3, 4]. Although, the use of antimicrobial agents has deeply reduced morbidity and mortality of patients, antimicrobial resistance increased substantially and is now a serious threat to public health [5-7]. Importantly, this high rate of antimicrobial resistance (mainly multidrug resistance) has a huge impact on patient's outcome due to failed treatments, which increases the hospitalization time, morbidity and even deaths [7-9]. Furthermore, economic outcomes are also associated [8, 9].

In industrialized countries, HAIs occurs in 5 to 10% of all hospital admissions becoming one of the most serious patient safety issues in modern healthcare [5, 7]. In 2012, the prevalence of HAIs in Portugal was 10.6%, the highest rate among European countries [10]. According to recent surveys, the annual economic impact of HAIs was approximately $9.8 billion, in the United States of America (USA) [6] and approximately €7 billion, in Europe (EU) [7]. Within the EU's healthcare systems, the overall prevalence of patients, with at least one HAI on any given day, was 5.7%. The most prevalent infections were respiratory tract infections (pneumonia and lower respiratory tract, 19.4% and 4.1%, respectively) that were in 33% of the cases, associated with a medical device. Surgical site infections (SSIs; 19.6%), urinary tract infections (UTIs; 19.0%), bloodstream infections (BSIs; 10.7%) and gastro-intestinal infections (7.7%) are also very common. UTIs and BSIs were reported as catheter-related in 59.5% and 39.5% of the cases,

respectively [7]. Portugal follows the same tendency than in the rest of Europe [10]. Moreover, Portugal shows the highest rate (85.7%) of central vascular catheter utilization in Europe and 31.3% was associated with HAIs [7, 10]. Central-line associated bloodstream infections (CLABSIs; 40%) were the most common in USA, followed by catheter-associated urinary tract infections (CAUTIs; 27%), SSIs (23%) and ventilator-associated pneumonias (VAPs; 10%) [6].

1.2. Clinical significance of coagulase-negative staphylococci

Previously considered as harmless inhabitants of human flora of the skin and mucous membranes with low virulence potential, coagulase-negative staphylococci (CoNS) are now acknowledged as important human pathogens [11, 12]. Despite the commensal trait, this group of gram-positive bacteria is highly prevalent in healthcare related infections worldwide, mainly device- or surgery-associated infection *(Table 1.1)* [13, 14]. Immunocompromised patients, long-term hospitalized and those with implanted foreign bodies are at major risk for CoNS infections [11, 15]. Moreover, evidence that CoNS species act as a reservoir for antibiotic resistance genes, may contribute to the evolution of CoNS as pathogens, thus increasing the problem of antibiotic resistance in staphylococcal infections [16]. CoNS isolated from HAIs had an overall frequency of 11.4% and 7.5%, respectively in USA and Europe, with higher incidence in bloodstream infection episodes [6, 7]. According to the 2012 report, in Portugal, the CoNS isolation rate was 4.5% increasing up to 32.6% for CLABSIs [10]. Similarly to Portugal, prevalence rate in CLABSIs was 34.1% and 32.1% in USA and Germany, respectively [13, 14]. The CLABSIs infection rate was higher in Canada (53%) and lower in Hungry (9.7%), during the same period [17, 18]. These data highlights the emergence of HAIs and indicates CoNS species as major pathogens in CLABSIs-associated morbidity worldwide.

Currently, 16 species of CoNS have been found in specimens of human origin and can be grouped according to the novobiocin susceptibility test. Twelve species including *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis* are susceptible to the antibiotic novobiocin and the other four species (*Staphylococcus cohnii*, *Staphylococcus saprophyticus*,

Staphylococcus sciuri and *Staphylococcus xylosis*) are novobiocin-resistant. *S. epidermidis* is by far the most predominant and persistent specie among CoNS clinical isolates, representing up to 80% of CoNS isolated from bloodstream cultures [15, 19, 20]. Importantly, *S. epidermidis,* the most isolated CoNS pathogen, is also, the most abundant commensal of human skin microbiota [21].

Table 1.1: Principal biofilm-associated microorganisms isolated from indwelling medical devices.

Indwelling medical devices	Principal Microorganisms
Central venous catheter	*Coagulase-negative staphylococci*
	Staphylococcus aureus
Mechanical heart valve	*Coagulase-negative staphylococci*
	Staphylococcus aureus
Urinary catheter	*Escherichia coli*
	Candida spp.
	Enterococcus faecalis
	Proteus mirabilis
	Coagulase-negative staphylococci
	Klebsiella pneumoniae
Artificial hip prothesis	*Coagulase-negative staphylococci*
	Enterococcus spp.
	Staphylococcus aureus
Artificial voice prothesis	*Candida spp.*
	Coagulase-negative staphylococci
Intrauterine device	*Staphylococcus epidermidis*
	Staphylococcus aureus
	Enterococcus spp.
	Candida albicans

(Adapted from Dufour *et al*. [22])

1.3. Pathogenesis of *S. epidermidis*

Despite de lack of aggressive toxins, *S. epidermidis* has others specific virulence factors *(Table 1.2)* mainly molecular factors, which make this opportunistic specie a successful pathogen. The biofilm-forming capacity is the major determinant of *S. epidermidis* virulence and, as a consequence, *S. epidermidis* has become of importance in the clinical setting [23-25]. In fact, *S. epidermidis* endogenous infections tend to be chronic due to bacterial ability to persist for a long time in the human body [25-27].

Table 1.2: Virulence factors of S*. epidermidis*.

Virulence factor	Gene	Function
Initial adhesion		
Autolysin adhesion	*aae*	Fibrinogen, fibronectin and vitronectin
Autolysin E	*atlE*	Binding to polystyrene and vitronectin; Affects surface hydrophobicity
Serine aspartate binding protein	*sdrF*	Binding to collagen
Fibrinogen binding protein (fbe)	*sdrG*	Binding to fibrinogen
Elastin binding protein	*ebp*	Binding to elastin
Extracellular matrix binding protein	*embp*	Binds to fibronectin
Teichoic acids	(a)	Binds to fibronectin
Biofilm accumulation		
Polysaccharide intercellular adhesin	*icaADBC*	Bacterial cell-cell interaction
Biofilm-homolog *S. aureus* protein	*bhp*	Intercellular adhesion (b)
Accumulation-associated protein	*aap*	Bacterial aggregation (requires proteolytic processing for activity) (b)
Extracellular matrix binding protein	*embp*	Bacterial aggregation
Teichoic acids		Component of biofilm matrix
Immune evasion		
Polysaccharide intercellular adhesin	*icaADBC*	Protects from IgG, AMPs, phagocytes and complement attack
Extracellular matrix binding protein	*embp*	Protects from phagocytes
Resistance to AMPs		
Metalloprotease/elastase	*sepA*	Involved in AMPs degradation
AMP sensing system	*apsRS*	Senses AMPS and resistance mechanisms
Toxins		
Phenol soluble modulin	*psmδ*	Pro-inflammatory cytolysin
Exoenzymes		
Lipases GehC, GehD	*gehC, gehD*	Persistence on human skin
Serine protease	*sspA*	Degradation of fibrinogen and complement factor C5
Cysteine protease	*sspB*	Tissue damage

Legend: (a) Multiple biosynthetic genes; (b) Independent from PIA. (Adapted from Otto *et al*.[28])

1.3.1. Biofilm formation

Biofilms are complex three dimensional structures described as a community of adherent microorganisms encased in a self-produced extracellular matrix, with protection as the primary function [29-31]. The extracellular polymeric substance (EPS) matrix consists in a complex mixture of polysaccharides, proteins, teichoic acids and extracellular DNA (eDNA) and other macromolecules [30]. EPSs are highly hydrated (98% water) thereby, preventing desiccation in some natural biofilms, and have adhesion propensity [30, 32]. Polysaccharides are major

components of EPS matrix, in contrast to eDNA which represent a minor fraction of *S. epidermidis* biofilm matrix [30, 33]. The EPS matrix plays a structural stabilizing role in biofilms, although it provides inherent protection against some antibiotics [34], disinfectants [35] and host-defense mechanisms mainly by neutrophil phagocytosis [29] contributing significantly to *S. epidermidis* biofilm survival *(see sub-sections 1.3.3. and 1.4.)*. It is generally accepted that the mechanisms underlying these protective features are several and might be different for antibiotic or host defense mechanism. Moreover, biofilms cells show considerable differences regarding gene expression profiles and physiology when compared to free-floating (or planktonic) bacteria counterparts [29, 30, 33]. The altered gene expression in *S. epidermidis* biofilms is characterized by a reduction of basic cell processes (such as, cell division, DNA transcription and protein synthesis) which are linked to the pathogenesis of *S. epidermidis* biofilm infection and to the protective mechanisms mentioned above [36].

The dynamic process of biofilm formation comprises three major and successive phases: *(I)* primary attachment of planktonic cells, *(II)* accumulation into multiple bacterial layers and maturation of the biofilm architecture and finally *(III)* detachment and dispersion of planktonic cells, which then may initiate a new cycle of biofilm formation elsewhere [29, 30, 33] *(Fig. 1.1)*. All three phases are crucial for biofilm-associated infections.

(I) Primary attachment: The first phase of biofilm formation is characterized by the attachment either to biotic surfaces (such as, tissues and/or conditioning films) or abiotic (such as, indwelling medical devices) and is mediated by both specific and non-specific mechanisms, respectively [29]. Indeed, primary attachment is a prerequisite for biofilm formation in indwelling medical devices.

The attachment to abiotic surfaces depends on physicochemical properties of the material used and on bacterial cell surface characteristics, and is driven mostly by hydrophobic and electrostatic interactions and reversible van der Waals forces [37, 38]. The *S. epidermidis* major surface-bound autolysin AtlE (148 KDa) is also involved in the primary attachment to polystyrene surfaces [39, 40]. Of note, AtlE protein contains two domains with bacteriolytic activity: an amidase domain (60 kDa) and a glucosaminidase (52 kDa) domain, but lacks the Leu-Pro-Xaa-Thr-Gly

(LPXTG) motif responsible for cell surface linkage and very common in others cell wall proteins [40]. AtlE, as other autolysins, has enzymatic and adhesive characteristics. Moreover, chromosomal DNA release from *S. epidermidis* (extracellular DNA) via the AtlE activity, was also found to be involved in primary attachment [41]. Two related staphylococcal surface-associated proteins – Ssp1 (280 KDa) and Ssp2 (250 KDa; a probable degradation production of Ssp1), were previously identify by Veenstra and coworkers, as contributors for this mechanism [42]. Although, recent findings suggested that, those proteins may be related to the accumulation-associated protein *i.e.*, Aap [43-45].

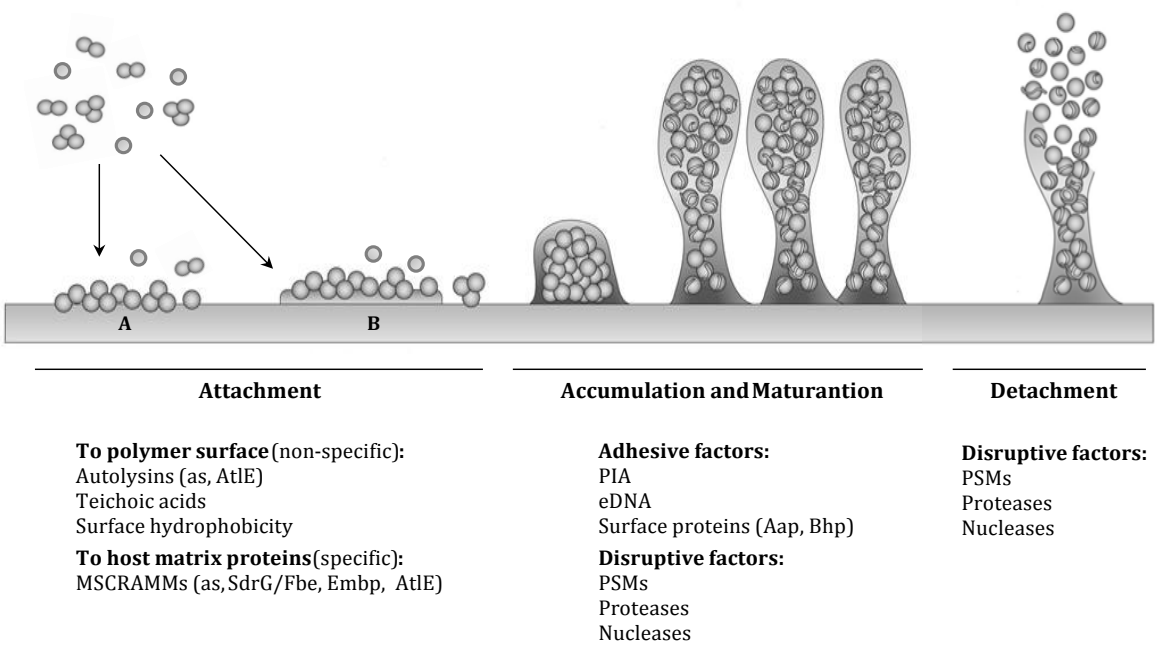

Figure 1.1: Representation of *S. epidermidis* biofilm formation and virulent factors involved in each phase. Attachment that occurs directly to uncoated surfaces (A) is mainly dependent on cell surface hydrophobicity, autolysins and teichoic acids, whereas bacterial attachment to coated surfaces as indwelling medical devices (B) is mediated via MSCRAMMs. After adhesion, biofilm accumulation and followed maturation is mediated by PIA, surface proteins, eDNA and also teichoic acids. The last phase is the detachment of biofilm cells that may leads to the colonization of new infecting sites in human body. (Adapted from Otto, 2009 [28])

Conversely, the attachment to a conditioning film composed of specific extracellular matrix (ECM) proteins (such as, fibrinogen, fibronectin, vitronectin, thrombospodin and collagen) and coagulation products (thrombi and activated platelets), might represent a more important mechanism of bacterial adherence and colonization [46]. The surface-associated adhesins designated as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)

accomplish binding of *S. epidermidis* to ECM proteins promoting the bacterial adherence to host tissues or matrix protein-coated devices [47]. The most important autolysin/adhesin is AtlE [40]. In fact, AtlE has a dual role in attachment as it promotes binding to uncoated polystyrene surfaces and to plasma protein-coated polymer surfaces [40, 48]. Similar to AtlE, the so called autolysin/adhesin from *S. epidermidis* (Aae; 35 KDa) show vitronectin-binding ability and bacteriolytic activity [49]. Other staphylococcal-binding proteins such as the fibrinogen-binding Fbe/SdrG (119 KDa) [50-53], the extracellular matrix binding protein (Embp; 1 MDa) [54, 55] and the lipase GehD (45 KDa) which promotes the attachment to collagen coated surfaces [56], also binds to ECM proteins and mediates adherence of *S. epidermidis* bacterial cells. Both *fbe* and *embp* genes were show to be widely distrusted in *S. epidermidis* clinical isolates [50, 57, 58].

In addition, purified teichoic acids – a source of polyanionic charge, have been shown to be involved in adherence to fibronectin [59]. Teichoic acids occur in two major forms; the lipoteichoic acids, which are linked to the cell wall, and the wall teichoic acids, which are covalently linked to peptidoglycan present in the biofilm matrix [60].

(II) Bacterial accumulation and maturation: This second phase of biofilm formation is characterized by the formation of multilayered bacterial clusters on top of the monolayer previously formed and followed by bacterial maturation.

So far, several molecules are responsible for this cell-to-cell adhesion but the polysaccharide intercellular adhesin (PIA) is the best studied [61-65]. Previously referred as capsular polysaccharide/adhesin (PS/A) [66, 67], the PIA molecule also known as poly-*N*-acetylglucosamine (PNAG) [68], is a major functional component of the biofilm matrix [61, 63]. The chemical structure of PIA was originally determined in 1996 by Mark and coworkers [62]. PIA is a homoglycan of *β*-1,6-linked *N*-acetylglucosamine (GlcNAc) residues and comprises two structurally related polysaccharides, a major polysaccharide I (80%) and a minor polysaccharide II (20%), with an average chain length of 130 *N*-acetylglucosamine residues [62]. On average, 80% of PIA molecule is *N*-acetylated, while the rest is non-*N*–acetylated and positively charged, which is essential for the functional activity of PIA [62, 69]. At the molecular point of view, production of PIA is encoded by the intercellular adhesin (*ica*) genetic locus [61, 63]. The *ica* operon *(Fig. 1.2)* is composed by four

open reading frames – *icaA*, *icaD*, *icaB*, and *icaC*, and the expression of all four *icaADBC* genes is required for a production of an active PIA molecule [63]. *IcaR* is found upstream of the *icaA* start codon and encodes a 185 amino acid (aa) tetR-type transcriptional regulator and negatively regulates the *icaADBC* transcription [70, 71]. IcaA (48 KDa) is an integral transmembrane protein with catalytic activity that works in tandem with IcaD (11 KDa) for full *in vitro N*-acetylglucosaminyl transferase activity as demonstrated by Gerke and coworkers [63]. Together, *icaAD* synthesized oligomers up to 20 residues of length [61, 63]. Moreover, *icaD* seems to act as a link between *icaA* and *icaC* genes [63]. IcaC (42 KDa) has a putative role in the elongation, externalization and translocation of the growing polysaccharide to the cell surface and is responsible for the synthesis of the full-length PIA molecule. IcaA, IcaC and IcaD are all transmembrane protein but in contrast to IcaA and IcaD, IcaC has no transferase activity [61, 63]. The expression of *icaADC* is a necessary requirement for the production of PIA. IcaB (34 KDa) is responsible for deacetylation of PIA, and crucial for PIA full activity in biofilm formation and virulence of *S. epidermidis* strains [69, 72]. In addition to its function in cell-to-cell adhesion, PIA is also responsible for the hemagglutinating activity of *S. epidermidis* [73, 74]. Of note, DNA sequences homologous to the *ica* locus were detected in a variety of CoNS species (such as, *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus saprophyticus*, *Staphylococcus lugdunensis* and *Staphylococcus intermedius*) as well as in *Staphylococcus aureus* [75, 76]. Many epidemiological studies have in fact, demonstrated that the presence of *icaADBC operon* is linked to the chronic and persistent nature of *S. epidermidis* biofilm infections [61, 77-80].

Although PIA synthesis is considered the predominant mechanism of biofilm accumulation in the last decade, many *S. epidermidis* clinical strains lacking the *ica* genes have been isolated at the clinical setting, suggesting the existence of additional factors independent from PIA, that could be involved in the pathogenesis of *S. epidermidis* [58, 81-83].

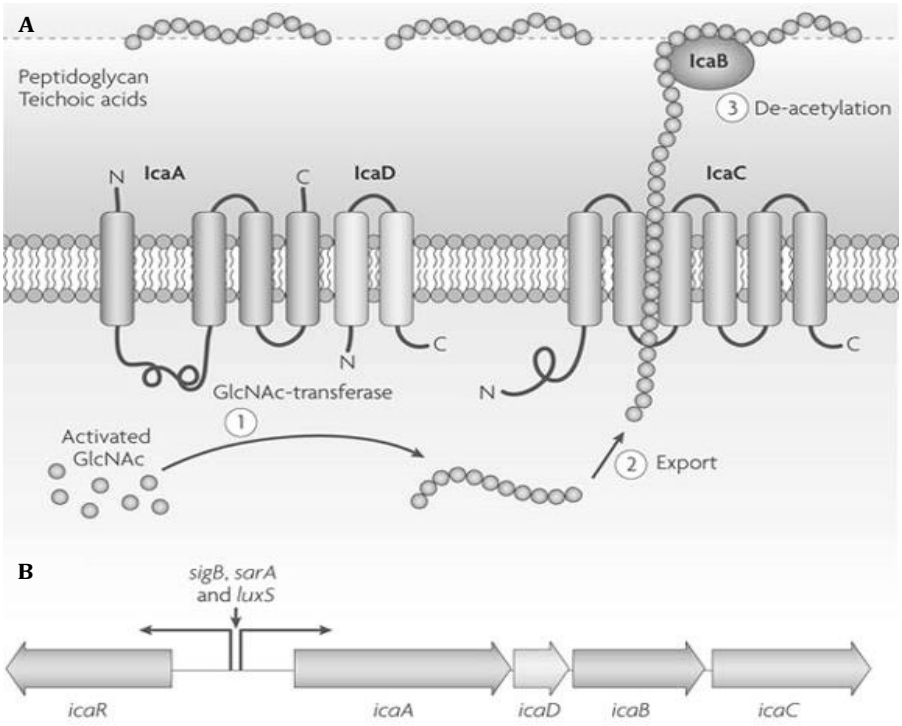

Figure 1.2: Representation of *S. epidermidis* Ica proteins and *icaADBC* gene. **A)** The PIA molecule is synthesized by the membrane-located GlcNAc transferase IcaA, which needs IcaD membrane protein for activity ①. The growing polysaccharide chain is exported by the IcaC membrane protein ②, then IcaB removes some of the *N*-acetyl groups, giving the polymer a cationic character that is essential for surface attachment ③. **B)** The Ica proteins are encoded by the *ica* gene locus containing the *icaADBC* operon and the *icaR* gene, which encodes a regulatory protein. Expression of the *icaADBC* operon is regulated either directly at the icaA promoter or through expression of IcaR, both of which are controlled by a series of global regulatory proteins (SigB, SarA and LuxS). (Adapted from Otto, 2009 [28])

Meanwhile, the Aap [84, 85] and the homologue to the biofilm-associated protein (Bap) of *S. aureus* protein (Bhp, biofilm-homologous protein) [86] were accomplished as mediators in the biofilm formation mechanism acting as proteinaceous intercellular adhesins. Aap protein (140 KDa) is anchored via a LPXTG motif and comprises two major domains. The domain A may itself contain a lectin-like domain and the repetitive domain B, consisting of 128 aa repeats [43, 44, 87]. The removal of the *N*-terminal domain A of *S. epidermidis* Aap protein via proteolytic activity leads to exposure of domain B and gives adhesive proprieties to the Aap. By this mechanism, Aap is able to promote biofilm accumulation [43, 85, 88]. The surface protein homologous to the Bap of *S. aureus i.e.* Bhp [89], was also associated with a PIA-independent mechanism of biofilm formation in *S. epidermidis* clinical isolates, even if less frequent than Aap [57, 90, 91]. Bhp (239 KDa)

is a protein composed of *N*-terminal signal sequence follow by three large domains: the domain A, domain B (with unknown function) and the domain C. The domain C is composed of 16 tandem repeats of 86 amino acids each. The C-terminal segment contains an LPXTG motif and a wall-membrane-spanning region [86, 89]. Nevertheless, furthers studies are needed to determine the Bhp impact in *S. epidermidis* biofilm development and pathogenesis. The presence of *aap* is now considered as *icaADBC operon i.e.*, an important virulence factor [45, 88, 92, 93].
Additionally, teichoic acids are not only key contributors to *S. epidermidis* adhesion but also, to colonization and inflammation process *(see section 1.4.)* playing a major contribution into staphylococcal pathogenesis [60, 94, 95].

(IV) Bacterial detachment and dispersion: It is believed that this last phase is crucial to the survival of bacteria, either due to nutrients limitation, or simply to the dissemination of *S. epidermidis* cells (individually or in clusters) that may lead to the colonization of additional infecting sites in the human body [96]. In general, detachment and dispersion occur in response to environmental and growing conditions or by a *sensu stricto* manner [97, 98]. Some researchers have also suggested that detachment is a self-control mechanism seems to regulate biofilm structure and thickening process, in order to achieve a balance [99-101]. Indeed, detachment is considered a "growth-associated phenomenon" [101]. This phenomenon is mediated through a combined effect between bacterial quorum sensing via *agr* system *(see section 1.3.2.)*, shear forces and biofilm strength [100-104]. Notwithstanding, the mechanisms underlying *S. epidermidis* biofilm detachment are still not fully understood.

1.3.2. Regulation of *S. epidermidis* biofilm formation

Staphylococcal biofilm formation is under the control of a complex network of signaling molecules that regulate either activation or inhibition of the expression of specific biofilm components [96, 105]. Watnick and his colleague Kolter [106], had compared biofilms to cities were microbes are "social organisms" able to communicate between then, in a process called quorum sensing (QS). These communities produce and secrete signaling molecules (the so called, autoinducers) which trigger a cascade of cellular responses regulating gene expression in a cell-density-dependent manner and enabling the bacteria to adapt

to changing environmental conditions [105, 107]. Additionally, autoinducers are involved in both intra- and inter-species communication [96, 107, 108]. Although more studied in *S. aureus,* the QS was implicated in all phases of biofilm formation and directly linked to *S. epidermidis* pathogenesis [96, 105]. Not less important, QS significantly impacts the interaction of staphylococci species with innate host defense, owing potential as therapeutic targets for control of staphylococcal infections [109]. In Staphylococci, two main QS systems have been identified and characterized: the *(I) agr* QS system [105, 110-112] and the *(II) luxS* QS system [113].

(I) agr QS system: The *agr* system is composed by RNAII and RNAIII units that are respectively regulated by P2 and P3 promoters *(Fig. 1.3.)*. RNAIII includes an open reading frame (*hld* gene) encoding the δ-toxin [105, 114]. Of note, it has been speculated that *S. epidermidis* δ-toxin – a member of the phenol-soluble modulins (PSMs) family, may prevent adherence to abiotic surfaces [115, 116]. Despite surfactant properties, the *S. epidermidis* δ-toxin is known to cause the lysis of erythrocytes by forming pores in the cytoplasmic membrane [117]. In *S. aureus*, the RNAIII molecule was found to regulate the expression of many genes encoding cell wall-associated proteins via the RNAIII-inhibiting peptide (RIP) [118].
Moreover, it is accepted that RNAIII is the effector molecule of the accessory gene regulation [118, 119]. Conversely, RNAII contains a sequence of four co-transcribed genes: *agrB*, *agrD*, *agrC*, and *agrA* [111, 120]. The *agrACDB operon* is organized in a two-component transmembrane transduction complex (AgrA and AgrC), a pro-signalling peptide (AgrD), and a membrane component (AgrB). The *AgrA* gene binds to two promoters and activate via an auto-feedback manner, the transcription of the *Agr operon*. The *agrB* and *agrD* gene are responsible for the production and externalization of autoinducing peptides (AIP) [105, 120]. AgrC detects the secreted AIP and activates the regulator protein AgrA which in turn up-regulates promoters P2 and P3 [105, 119]. So, *AgrA* directly activates the expression of PSMs, a group of amphipathic peptides with pro-inflammatory properties encoded by the *hld* gene [121-123]. PSMs are important in producing mature biofilm structures and causing biofilm detachment [36, 124, 125]. The production of *agr*-regulated proteases and DNAses also has an important effect on staphylococcal biofilms development [105, 109]. Many studies have pointed out that the *agr* system plays a central role in staphylococcal pathogenesis [105, 109, 115, 126]. However, more

recent *in vivo* studies suggest that this role may be subtle [127-130]. Among the regulation of the expression of toxins (as, δ-toxin), cell surface proteins (as, MSCRAMMs) and adhesins (as, AtlE), the *agr* system also controls the interaction with the host immune system *(see section 1.4.)* [96, 130]. In addition, *agr* QS system is also associated to bacterial adaptation to different environmental situations in a more general way [131].

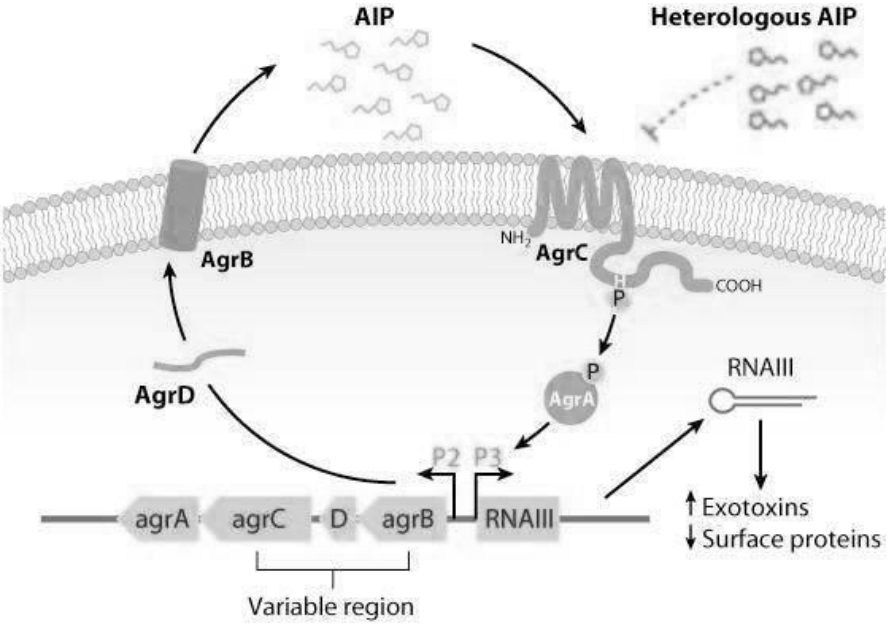

Figure 1.3: Representation of *S. epidermidis agr* QS system. ArgD encodes a peptide, which is processed and secreted by AgrB. Mature AIP interact with the histidine sensor kinase AgrA, leading to the phosphorylation of the response regulator ArgC, which in turn activates transcription from the two *agr* promoters. The P2 promoter drives the auto-activation circuit, and the P3 promoter drives transcription of the regulatory RNA (RNAIII), which is the regulatory effector of the system. (Adapted from Novick and Geisinger [132])

(II) luxS QS system: This system uses the LuxS/autoinducer-II (LuxS/AI-II) signal, which acts as a "universal signal" mainly in inter-species communication [113, 133]. Although the most spread QS system, an *in vitro* study carried by Lin and coworkers [134], suggested that *luxS* gene has no implication in *S. epidermidis* biofilm development. However, *luxS* has been linked to the decreases of bacterial accumulation hence, blocking the production of PIA via down-regulation of *icaADBC* operon [135].

Of note, down-regulation of the QS systems might enhance the success of *S. epidermidis* as a pathogen through the increased production of colonization factors, biofilm formation and reduced activation of the host immune response [105, 126].

1.3.3. Antimicrobial resistance

Another important virulence factor of *S. epidermidis* is the resistance to antimicrobial agents. This bacterial resistance can be either innate (also referred as intrinsic resistance) or acquired [35, 136].

The acquired antimicrobial resistance is closely related to the widely use of antimicrobial agents in the clinical practice and especially due to prolonged therapy [137, 138]. Acquired resistance occurs mainly by gene mutation and horizontal transfer of genes or either by combination of both [136, 139]. The process of horizontal transfer of genes relies on acquisition of exogenous genes via conjugation (plasmids and transposons), transformation (incorporation of chromosomal DNA released and/or plasmids from other bacteria) and transduction (bacteriophages) [136, 139]. Conjugation is the most common mechanism in horizontal gene transfer and acts as a key intervenient in the dissemination of antibiotic resistance mainly multidrug resistance [136, 137]. As mentioned before, Staphylococci species are notorious for evolving and spreading antibiotic resistance mechanisms, impairing treatment and patients' quality of life [35, 137]. The most prevalent mechanism of acquired resistance identified in Staphylococci with clinical importance is the inactivation of *β*-lactams mainly penicillin antibiotics. Methicillin – a type of penicillin antibiotic was for many years, an antibiotic of first choice against staphylococci [15, 140]. Nowadays, approximately 80% of *S. epidermidis* clinical isolates are resistant to methicillin and not less important, 30 to 40% of community isolates obtained from healthy individuals also show to be resistant to methicillin [140-144]. Methicillin-resistance in staphylococci is caused by expression of penicillin-binding protein 2 (PBP2a) which has low affinity for all β-lactam antibiotics [145]. PBP2a is a high-molecular weight class B trans-peptidase that catalyzes the formation of peptidoglycan and is encoded by the *mecA* gene that is located on a mobile genetic element called the staphylococcal cassette chromosome (SCC) [145, 146]. Maximal expression of resistance by PBP2a requires the efficient and correct

synthesis of the peptidoglycan precursor [146]. There is evidence of horizontal transfer of SCC cassettes between staphylococcal species which implies that CoNS could serve as a reservoir for the spread of resistance genes [143, 147]. Five different types of SCCmec (I to V) have been described by the particular combination of two parts: the cassette chromosome recombinase (ccr) gene complex and the *mec* complex [148-150]. Only the SCC*mec* types I, IV and V do not contain any antibiotic resistance genes [148]. To date, 11 different structural elements of SCC*mec* have been observed in methicillin-resistant *S. epidermidis* (MRSE) [151]. In addition, several epidemiological studies have pointed out the high prevalence of SCC*mec* type IV among both clinical and commensal *S. epidermidis* strains worldwide [152-157].

Despite the importance of *S. epidermidis* acquired antibiotic resistance the innate resistance also plays a crucial role in the widespread of antimicrobial resistance. Furthermore, both acquired and innate resistances are closely linked to the increased pathogenesis of device-related biofilm infections.

Often the best treatment to device-related infections, is the removal of the infected device, but in the case of joint prothesis implants or biofilm growth on host tissue, implants or tissue removal is not always the most appropriate clinical attitude [158]. In those cases, antimicrobial therapy is the treatment choice. Of concern, sessile populations are up to 1000-fold more resistant to antimicrobial agents than planktonic counterparts [159]. As mentioned earlier, EPS biofilm matrix can act also as an active barrier limiting antibiotic diffusion [34, 35]. Notwithstanding, in some *in vitro* studies antibiotics such as rifampin, vancomycin, and daptomycin, have been shown to penetrate and diffuse easily into the *S. epidermidis* biofilm matrix [160, 161] even if some fail to kill sessile bacteria [160]. Interestingly, most *S. epidermidis* isolates are still susceptive to those antibiotics. Facing the widespread use of antibiotics, resistance has raised and is currently regarded as a major concern worldwide *(Fig. 1.4.)* [6, 7].

Two others intrinsic mechanisms are also associated to EPS biofilm matrix: the presence of specific resistance mechanisms as the deployment of adaptive stress responses, and the so called, dormant and persister biofilm cells [162-165]. So, while some bacteria may activate stress responses, others may differentiate into a protected phenotype. Dormancy is characterized as a decrease of basic cell metabolic processes such as, DNA replication or protein translation [162]. It has

been assumed that, cells under a physiological “dormant” state are more prone to survive under stress conditions. Moreover, this specific process decreases the efficacy of many antibiotics, mainly those that target cell wall activity and protein synthesis [35]. On the other hand, persister cells are recognized as cells that “neither grow nor die” in the presence of antimicrobial agents [162]. In fact, persister cells showed to be extremely tolerant to high concentrations of antibiotics highlighting their contribution to antibiotic resistance [162, 166]. Bacterial cells that persist under antibiotic treatment tend to repopulate the biofilm thereby, resulting in recalcitrant infections [162, 166]. While significant, persister cells represent a small portion of the biofilm population [162]. Both dormant and persister cells, not only prevent bacterial death by reducing the efficacy of many antibiotics but also elicit a reduced host inflammatory response *(see section 1.4.)* [36, 167]. These distinct phenotypes by themselves can confer an intrinsic resistance to some antimicrobial classes, including resistance to penicillin, quinilones and glycopeptides [34, 137, 168].

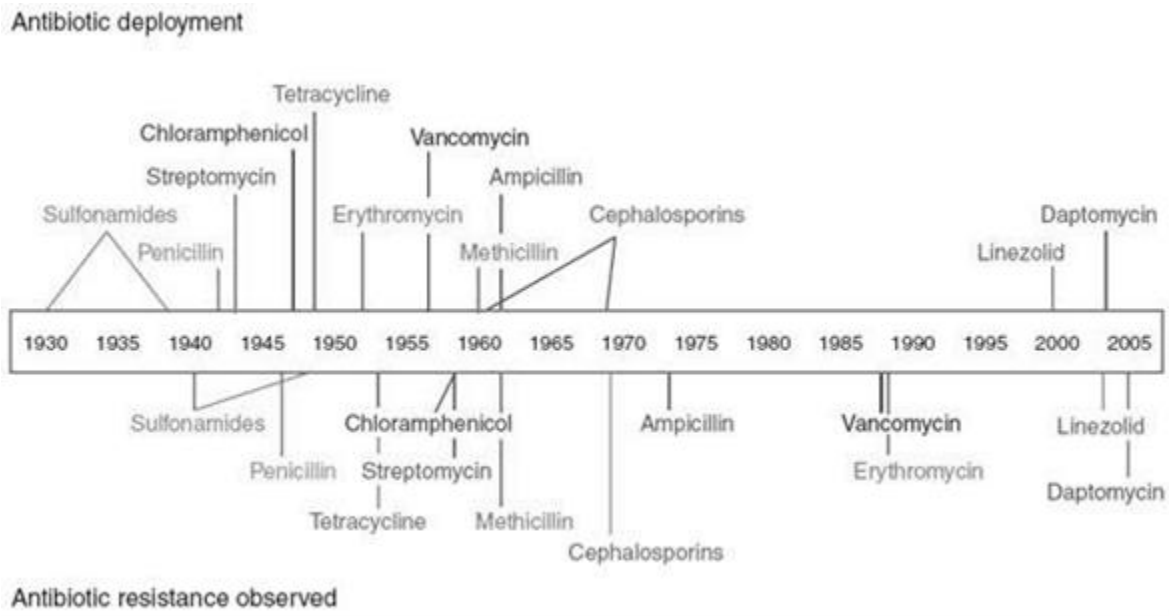

Figure 1.4: Timeline of antibiotic deployment and the evolution of antibiotic resistance. (Adapted from Clatworthy *et al*., 2007 [169])

In order to understand the way how resistance develops, it is first important to understand how each antimicrobial agent acts. Antimicrobial agents are often categorized according to their principal mechanism of action that include inhibition of cell wall synthesis and of cell membrane function, inhibition of protein synthesis,

interference with nucleic acid synthesis and inhibition of a metabolic pathway [35, 170]. Some of the most important antibiotics with clinical use for treatment of *S. epidermidis* infections are described below. Importantly, evidences about the decline interest in antimicrobial drugs discovery by commercial entities are of major concern [171, 172].

Cell membrane and cell wall-active antibiotics

Most of the glycopeptides-resistant clinical isolates are resistant to teicoplanin, but susceptible to vancomycin [173]. Vancomycin is a glycopeptide that binds to the peptidoglycan side chains in the cell wall, preventing cross-linking during cell wall synthesis [174]. In fact, the cell wall is the target of this antibiotic. Its effect leads to a weakened cell wall, slowing growth and eventually causing death of bacterial cells. The efficacy of vancomycin treatment for *S. epidermidis* biofilm infections has been evaluated and susceptibility rates to vancomycin were found to be around 60 to 100% in clinical isolates [160, 175]. Many studies had shown that the treatment of biofilms with vancomycin had increase its efficacy if in combination with other antibiotics (rifampicin, gentamicin, clindamycin) and also had shown it led to a better penetration of the drugs into the biofilm increasing cell death [160, 176, 177]. Despite some increased resistance, vancomycin remains a treatment of choice for invasive staphylococcal infections [178]. Moreover, novel antibiotics have been developed to overcome the difficult situation of vancomycin, considered the last effective antibiotic against many multi-resistant strains [178].

Daptomycin is a relatively new lipopeptide drug, with proven successful against several forms of Gram-positive infections [179-182]. This bactericidal agent consists of a 13-member amino acid cyclic lipopeptide with a decanoyl side-chain that confers a unique mode of action [183, 184]. The lipophilic daptomycin tail binds to the bacterial membrane, in the presence of physiological levels of calcium ions causing disruption of membrane structure [183-185]. This is followed by arrest of DNA, RNA and protein synthesis resulting in bacterial cell death [183, 184]. For treatment of biofilm infections, daptomycin alone is more effective than other monotherapies used to combat staphylococcal infections [186]. Due to mechanism of action, daptomycin appears to be an excellent therapeutic option against staphylococcal infections [161, 179, 181, 182, 186-188].

Inhibitors of DNA and RNA synthesis

Rifampin or rifampicin is an inhibitor of DNA and RNA synthesis. It targets the bacterial RNA polymerase and inhibits RNA synthesis [189]. Several *in vitro* studies, it has been observed that rifampicin is surprisingly effective against staphylococci biofilm. In *S. epidermidis*, the antibiotic effectively penetrates the biofilm layers, preventing biofilm formation [160, 190, 191]. Owing to increased resistance, rifampicin if used in the treatment of catheter-related infections must be used in combination with other antimicrobials or dispersal agents [192-195]. Moreover, due to intrinsic mechanisms, *S. epidermidis* easily become resistant to rifampicin by accumulating mutations in the RNA polymerase β subunit (*rpoB*) gene, which encodes the β subunit of bacterial RNA polymerase [35, 196]. Currently, resistance has remained at a relatively low level but is a significant concern for all staphylococcal infections [7].

Inhibitors of protein synthesis

Oxazolidinones are a new group of synthetic drugs that have demonstrated activity against *in vivo* and *in vitro* bacterial biofilms and also, against methicillin- and vancomycin-resistant *S. epidermidis* strains [197, 198]. Oxazolidinones inhibit ribosome function and prevent protein synthesis by disrupting assembly of ribosomal subunits, owing a unique mechanism distinct from other ribosome-targeting drugs [197, 199]. Linezolid is the first oxazolidinone available and so far, the only approved for clinical use [200].

1.4. Host immune response to *S. epidermidis*

As previously mentioned, biofilms are a physical result of adaptive features by many bacterial species, including *S. epidermidis*. Notwithstanding, the physiological changes in *S. epidermidis* biofilms that protect the bacteria from antimicrobial agents and enable the bacteria to persist during infection, also protect from the host immune system by phenotypic shifting to a non-aggressive state, reducing inflammation and the chemotaxis of immune cells to the site of infection [36, 201, 202].

The innate immune system is the human body's first-line of defense against invading microorganisms [203]. Among other, it includes three important mechanisms that enable killing of bacteria: secretion of antimicrobial peptides (AMPs) [203, 204] and complement activation followed by neutrophil mediated killing

[203, 205]. Serum complement coats the bacteria and then the opsonized bacteria are uptaken by the host “professional” phagocytes [203, 205, 206]. The complement system can be activated by three pathways that differ in their mode of recognition [205]. The classical pathway is activated by C1q lectin either by direct recognition of *S. epidermidis* surface structures or by binding to surface-bound antibodies. The lectin pathway is activated through binding of mannose-binding lectin to various carbohydrate residues. The alternative pathway is activated spontaneously, but will only proceed if bacterial surfaces are present. This pathway may also act as an efficient amplification loop for both the classical and lectin pathways. All pathways converge at C3 [205]. Furthermore, killing is also achieved by a vast array of proteases, lipases and amidases, and by nutrients limitation (such as, iron acquisition), or by a synergic combination of all of these factors [207]. Moreover, AMPs – an evolutionary ancient means of defense from microbial pathogens, are peptides that are present in, and secreted by neutrophils (and other cell types, such as epithelial cells) and act by killing bacteria through recognition and disruption of bacterial membrane integrity [204, 208, 209].

As a successful pathogen, *S. epidermidis* likely has means to evade host innate immune recognition. Both biofilm formation and PIA synthesis play central roles in the evasion of host immune defenses by protecting *S. epidermidis* from PMN phagocytosis, AMPs and antibody recognition [69, 210-212]. In addition, the *S. epidermidis* protease SepA (33 kDa), via proteolysis [209] and teichoic acids, via molecule structural changes [204] also contributes to the AMPs inactivation. Furthermore, the *agr* QS system is also implicated in the regulation of *S. epidermidis* specific immune mechanism of evasion [208, 209]. Of note, *S. epidermidis* PSMs (specifically the δ-toxin) have a more pro-inflammatory role like PMNs activation and cytokine release, than capacity to lyse neutrophils [206]. Combined these findings, largely contributes to the understanding of the extraordinary success of *S. epidermidis* as colonizers and infective agents on human. Furthermore, it is clear that as a commensal organism living on the human skin, *S. epidermidis* has a more passive defense strategy rather than using aggressive toxins [206].

1.5. Anti-biofilm therapeutics

Facing the increased resistance to antimicrobial agents, novel strategies to inhibit bacterial attachment and/or colonization of indwelling medical devices by CoNS in general, are needed. These strategies include the development of novel antibiotics. For instance, daptomycin, quinupristin/dalfopristin and those belonging to the oxazolidinone group (such as, linezolid) are included in the novel group of antibiotics developed to overcome the resistance problem in CoNS isolates [137]. In an effort to combat biofilm infections associated with medical devices impregnated devices with antibiotic or antiseptics that could prevent bacterial colonization and also reduce the use of systemic antibiotics have been explored [26, 213]. Moreover, these surfaces are inexpensive, have long shelf-lives and most important, do not affect the overall function of a device which are major advantages [214]. The application of topical antibiotics at catheter insertion sites has been also shown to prevent staphylococcal infections [213]. Importantly, a good antibiotic policy in order to reduce the antibiotic pressure will also help to reduce the incidence of multi-resistant CoNS isolates. Additionally, natural substances as farnesol [215, 216], tea tree oil [217] and eucalyptus oil [218] can act as antimicrobial adjuvants or even serve as potential alternatives to antibiotics, impairing biofilm growth.

Another common strategy is to weaken the biofilm structure by degradation of the matrix [219]. Polysaccharide, eDNA and proteins, are the principal components of EPS matrix. Hence, targeting these components via enzymes that degrade each of these molecule, such as polysaccharide hydrolases (as dispersin B) [220, 221], proteases [222] and/or DNAses [223, 224], can disassemble staphylococcal biofilms. Degradation of the matrix eventually leads to biofilm bacterial dispersion. Once dispersed, they shift to a planktonic stage and regain more sensitivity to antibiotic treatment [104, 225]. This combination approach of matrix-degrading agents with antibiotics could be very promising. Additionally, the enzyme lysostaphin that damage the staphylococcal cell wall detaching biofilms cells appears to be a promising agent against *S. epidermidis* infections [226]. Moreover, small-molecule inhibitors of basic cell processes like DNA, RNA, and protein synthesis, are some of the most effective agents against staphylococci biofilm formation either by inhibition or disruption of the biofilm EPS matrix [35].

In addition, methods blocking the expression of genes that control *S. epidermidis* adherence or accumulation (such as, *icaADBC* and *aap*) to medical devices might help to prevent this type of infection. Approaches for targeting the *agr* QS through RIP molecule are also possible and promising [118, 227, 228]. Despite slower lytic performance, bacteriophages can also be useful as therapeutic agents against *S. epidermidis* biofilm infections [229, 230].

1.6. Aims of this study

S. epidermidis is a commensal inhabitant of the human flora of the skin and its pathogenicity is mainly due to the ability to form biofilms on indwelling medical devices. In fact, biofilms are a protective mode of growth. Due to adaptive mechanisms, *S. epidermidis* is highly protected against attacks from the host immune system and antibiotic treatment, making *S. epidermidis* infections difficult to eradicate impacting patients' morbidity and mortality [29, 36, 159]. Moreover, that unique feature seems to be closely related to either commensal or pathogenic *S. epidermidis* lifestyle [12, 26]. The physiology of biofilm development appears to be very complex and the understanding of how some molecular determinants affect the process of biofilm formation and development in *S. epidermidis*, remain uncertain. For instance, the impact of *icaA*, *aap* and *bhp*, in the biofilm formation of isolates from different origins is still poorly understood.

Therefore, this thesis has as its primary objectives:

I. To characterize molecular and phenotypically a group of Portuguese *S. epidermidis* isolates recovered from patients diagnosed with device-associated infections;
II. To *in vitro* study the interactions between *icaA*, *aap* and *bhp* genes and their relative contribution in different stages of biofilm development, in a subset of clinical and commensal *S. epidermidis* isolates.

CHAPTER 2

OPTIMIZATION OF *S. EPIDERMIDIS* BIOFILMS QUANTIFICATION AND TRANSCRIPTIONAL ANALYSIS TECHNIQUES

This chapter describes an optimization process of two techniques used during the course of this thesis.

2.1. Introduction

The emergence of *S. epidermidis* as an infectious agent has been correlated with the growing number of immunocompromised patients and with the widespread use of indwelling medical devices [29, 231]. *S. epidermidis* has a preferential ability to adhere to surfaces and to survive as a biofilm [24, 231]. This biofilm-forming ability plays a key role in the pathogenesis of *S. epidermidis* and has been associated with many hospital-acquired infections, affecting patients' treatment and increasing the costs [3, 232, 233]. In fact, the biofilm "mode of survival" brings major benefits since not only does it provide structural support to the bacteria, as it also protects them from the host immune system [206, 212] and antimicrobial agents [34, 234].

Due to the impact of biofilms in health, a broad range of techniques have been described for specie identification and to *in vitro* study the biofilm formation. However, accurate methods to quantify the total amount of biofilm *i.e.*, bacterial cells plus extracellular matrix, are still challenging. For instance, the diagnosis of catheter-related bloodstream infection, is made by semi-quantitative roll plate method [235] and by disruptive methods, as sonication and vortexing preceding plate counting [236]. Notwithstanding their clinical usage, these methods involve the culture from the catheter tip and subsequent quantification of the number of colony-forming units (CFU), which is time consuming. Moreover, CFU evaluation has been described as suffering from a lack of reproducibility [237, 238] and can lead to significant errors due to the presence of cell clusters promoted by the biofilm matrix net. Colorimetric methods such as those using crystal violet (CV) or safranin stains, and optical density (OD) measurement of bacterial cultures are also common, easy and straightforward quantification techniques [239-241]. While very useful for screening purposes [242, 243], these methods have major limitations, as they tend to underestimate or overestimate the bacterial count. In order to overcome this issue, a homogeneous biofilm cell suspension is of major importance. Homogeneous suspensions are achieved by vortexing or sonication, two methods that demand some optimization and raise some concerns. Studies focusing on the elimination of biofilms from infected medical devices have clearly demonstrated that either vortexing or scraping do not offer sufficient biofilm disruption [244, 245]. Conversely, sonication contributes to a better dispersion, making the culture after sonication easier to quantify [245-247]. Nevertheless, the issue of

biofilm maturation has not been taken into account. As we have previously shown [248], biofilm formation is a dynamic process and mature *S. epidermidis* biofilms are often associated with higher biomass and higher expression levels of adhesins [23], thus being more complex to evaluate and/or to eradicate.

Nowadays, microscopic examination is an accepted gold standard not only for biofilm detection and quantification, but also for evaluation of the biofilm formation process [249-252]. In particular, fluorescence microscopy approaches based on fluorogenic dyes offer detailed insights into bacterial biofilms, and have been considered as a sensitive and accurate methodology [250, 253, 254].

In addition to the mentioned methods that rely on traditional approaches, advances in molecular technologies – as polymerase chain reaction (PCR) and quantitative real-time PCR (pPCR) – have been introduced over the last decades and revolutionized our ability to rapidly detect and identify microbial organisms [255, 256]. Indeed, these genetic methods bring major advantages for the quantification of fastidious or very slow growing microorganisms [257]. For instance, the qPCR is an effective and sensitive tool used to investigate the pathogenic role of bacterial biofilms [258-260]. Nevertheless, this method also has some drawbacks related to biofilm sample preparation, mainly due to high content of proteins and polysaccharides in the extracellular matrix. Other limitations are the number of genes that can be simultaneously analyzed and the associated high costs [261]. Despite the methods used for quantification, visualization and characterization of microorganisms have undergone rapid progress, the results are not linear, and depending of the type of microorganism involved, further optimization is still needed.

In order to obtain optimal results the work developed in the scope of this chapter aimed at exploring conventional methods to achieve a high-throughput quantification of *S. epidermidis* bacterial biofilms.

In the *sub-chapter 2A*, the effect of sonication on the elimination of *S. epidermidis* cell clusters from biofilms developed over time was addressed and the accuracy of the three quantification methods was evaluated. Furthermore, a fluorescence microscopy automatic counting technique was optimized. In the *sub-chapter 2B*, a custom RNA extraction assay was described and the efficacy among different commercial reverse transcriptase kits and qPCR master mixes was assessed.

This optimization comprises minimization of time and costs, and maximization of reproducibility and sensitivity.

2.A. EFFECTS OF SONICATION ON THE ELIMINATION OF *S. EPIDERMIDIS* CELL CLUSTERS FROM BIOFILMS GROWN OVER TIME.

2.A.1. Material and Methods

Bacterial strains and growth conditions

Three well-known biofilm-forming strains were used in this study: *S. epidermidis* RP62A (PubMed accession number: PRJNA57663, ID: 57663), *S. epidermidis* 9142 [262] and *S. epidermidis* 1457 [263]. Biofilm cultures of each strain were performed in fed-batch mode as previously described [248]. Briefly, a starter culture was grown overnight in Tryptic Soy Broth (TSB) (Oxoid) at 37 °C with agitation (120 rpm, 10 mm orbit diameter, Biosan). In a 24-well plate (Orange Scientific), a 100-fold dilution was performed in TSB supplemented with 1% (w/v) of glucose (TSBg) to induce biofilm formation. The cultures were grown in the same conditions for 24, 48, or 72 h. The growth medium was completely removed and replaced using an equal volume of fresh TSBg every 24 h. Each experiment was repeated at least three times.

Biofilm disruption

After each incubation time, the biofilm was washed twice, removed from the plate by scraping and resuspended in 1 mL of a physiological saline solution (NaCl 0.9%). Afterwards, biofilm suspensions were sonicated (Cole-Parmer® 750-Watt Ultrasonic Homogenizer, 230 VAC, employing a 13mm microtip) using three different cycles differing in time (sec) and amplitude (%): *cycle A* – 10 sec at 30%; *cycle B* – 30 sec at 30% plus 40 sec at 40%, and *cycle C* – *cycle B* plus 120 sec at 40%. The tubes containing the biofilm samples were kept in ice during sonication. The scraping procedure removed more than 98% of the biomass, determined by the reduction in crystal violet staining. A cell suspension vortexed for 1 min (WS) was used as the no-sonication control, as previously described by Olson *et al*. [264].

Biofilm quantification

Biofilms were quantified using three distinct methods routinely used in microbiology.

I. To determine cell viability, a single biofilm was resuspended in 1 mL of NaCl 0.9%, followed by sonication or vortexing as described above. Several serial 10-fold dilutions were made in saline solution and plated on Tryptic Soy Agar (TSA). The plates were incubated at 37 °C for 24±2 h before counting the number of CFU.

II. Biofilm biomass quantification was done by measuring the OD_{595mn} of each biofilm suspension. Biofilm suspensions were diluted to bring OD into the linear range between 0 and 0.8 absorbance units. The measured OD_{595mn} was then multiplied by the dilution factor.

III. Total and dead cells were quantified using a Neubauer chamber coupled with an Olympus BX51 epifluorescence microscope equipped with a CCD color camera DP71 (Olympus). Biofilm suspensions were stained with the commercially available LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) following the manufacturer's instructions. A negative control was used to determine the baseline threshold for dead cells. Cells were then counted at a magnification of 200x using the automated enumeration software SigmaScan Pro 5.0 (Systat Software Inc.) as described before [265]. Briefly, 20 TIFF images (1360 x 1024) per condition were acquired and converted to eight bit 256 grayscale, to be analyzed through the differences in the gray intensity of each pixel, by using an appropriate intensity threshold experimentally determined. Under these conditions, 18420 x 1575 pixels were equivalent to 0.0025 cm^2 at 200x magnification.

Statistical analysis

The assays were compared using one-way analysis of variance (ANOVA) by applying the Tukey's multiple comparisons test and the paired sample t-test, using SPSS. All tests were performed with a confidence level of 95%.

2.A.2. Results and Discussion

Automatic image counting validation

Automatic image counting software can be a useful tool in research laboratories [266, 267], but care should be taken to guarantee that the selected software is accurately quantifying the desired object of study. To validate the SigmaScan Pro 5.0 software for automatic counting of *S. epidermidis* biofilm cells, using the fluorescence-based Live/Dead staining, several and different parameters were tested. Firstly, we tested the intensity threshold settings of the software, since it has been previously shown that different fluorophores can yield different quantification of bacteria [254]. To achieve that, several images were manually counted by an independent user. Each image was then processed by the software by another independent user, without previous knowledge of the manual counting results. Three analyses were performed using high, medium and low intensity threshold. As illustrated in the *figure 2.A.1A*, no significant differences ($\rho > 0.05$) were found using any of the three software thresholds and the manual counting. This data indicates that the Live/Dead staining was strongly discriminative between bacteria and background and that there was no significant fluorophore bleach effect that could impair the automatic counts. We have tested the sensitivity of the method by using a magnification of either 200x or 400x. Using a 200× magnification would not be appropriate for manual counting of *S. epidermidis* cells as it would be nearly impossible to discriminate between the background and individual cells, or even between individual cells and small cell aggregates. However, as illustrated in the *figure 2.A.1B*, microscopy quantification of *S. epidermidis* cells using a magnification of 200x is achievable. A further advantage of using the magnification of 200x is that with the same amount of images, the double of the surface area (as compared with the 400x) can be analyzed. Lastly, to discriminate between the two different fluorophores present in Live/Dead staining, the total number of live and dead bacteria was determined either by manual or automatic counting, using 200x magnification and a medium intensity threshold (*Fig. 2.A.1C*). Both fluorophores were correctly discriminated by the software, validating our automatic counting system.

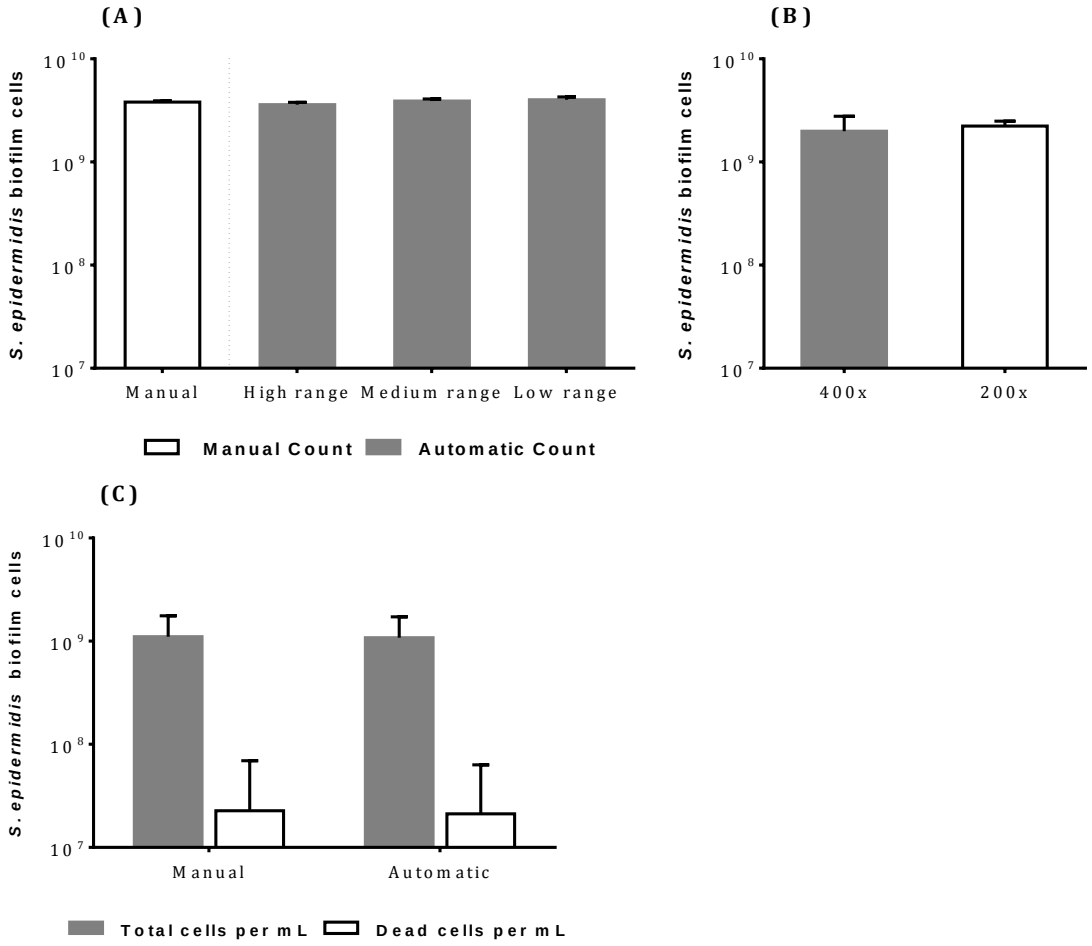

Figure 2.A.1: Validation of SigmaScan Pro 5.0 software: **(A)** Effect of the intensity threshold range determination in bacteria quantification, as compared with manual counting. **(B)** Manual versus automatic counting of total and dead cells obtained by using Live/Dead staining. **(C)** Bacterial quantification by automatic counting using a magnification of 400x or 200x. The values represent the mean ± standard deviation of three independent experiments. No significant changes were found ($\rho > 0.05$).

The effect of cell agglomeration in bacterial quantification

S. epidermidis is known to adhere to multiple surfaces and subsequent cell to cell aggregation allows the establishment of biofilms [237, 268]. Since biofilm formation is considered a major virulence factor of *S. epidermidis* [231], many studies addressed the optimization of methodologies to detach bacteria from infected medical implants [236, 246, 247]. Sonication is one of the methodologies used [247, 269, 270], which have been shown to improve the sensitivity of molecular diagnostic assays, as PCR [271, 272].

In order to address the sonication impact in the viability and quantification of *S. epidermidis* biofilm cells, three different sonication conditions were tested and

analyzed by others three conventional used methods: (I) quantification of viable cells by CFU plate counting, (II) biomass quantification by OD_{595mn} measurements and (III) quantification of total cells by fluorescence microscopy analysis.

The OD measurements showed a progressive biomass accumulation during the analyzed time course of biofilm formation, as anticipated (*Fig. 2.A.2*). Importantly, the different sonication cycles resulted in significant changes in the OD measurements in the biofilms with 48 or 72 h of grow. However, in 24 h-old biofilms, no differences were found between the three sonication cycles ($\rho > 0.05$) while in more mature biofilms (48 and 72 h of growth), the tested sonication conditions progressively influenced biofilm biomass quantification ($\rho < 0.05$) (*Fig. 2.A.2)*. Taking into consideration that the linearity of OD determination requires homogeneous suspensions, the detected variations suggest the presence of cell clusters and pointed out that sometimes a stronger sonication cycle is essential to break down those remaining clusters.

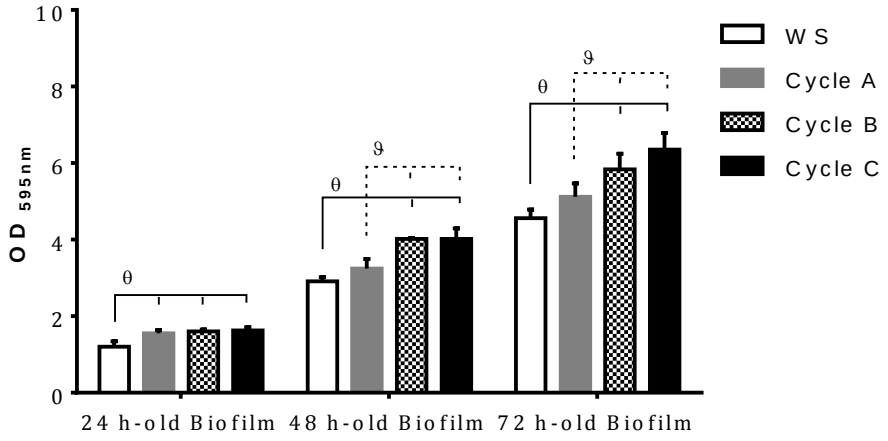

Figure 2.A.2: Bacterial cells quantification by optical density (OD_{595nm}) in biofilm suspensions grown over time, following vortexing (WS) or sonication (Cycle A, B and C). The values represent the mean ± standard deviation of three independent experiments. Statistical differences ($\rho < 0.05$) between no-sonication control (WS) and any other sonication cycle (θ) and, between the sonication cycle A and the others sonication cycles (ϑ) were analyzed with ANOVA Tukey's test.

Despite the widespread usage, low cost and quickness of this approach, quantification of biofilm cells by optical density does not provide accurate insights about the total number of bacteria. To address this issue, two other quantification methods were tested. Not surprisingly, cell quantification using the microscope approach allowed the detection of higher number of *S. epidermidis* cells in biofilms

after 48 or 72 h of growth, comparing to CFU plate count (*Fig. 2.A.3*). This fact can be explained by the ability to accurately differentiate between a cell cluster and an individual cell, which would otherwise be considered indistinguishable by CFU counting. Only at these later incubation periods, significant differences between sonicated and no-sonicated samples were detected ($\rho < 0.05$). This confirms that, in certain conditions, intense vortexing can be used instead of sonication. As a vortex is more affordable than a sonicator, some researchers might choose this option. However, it was clear that 1 min of vortex was unable to reduce the cell clusters size formed in mature biofilms pointed out that, sonication is a more effective treatment even at reduced time and lower frequency. Also, the longest time and sonication intensity used (cycle C) did not affect the viability of *S. epidermidis* biofilm cells, as validated by live/dead microscopic observations and culturability, as determined by CFU (*Fig. 2.A.3*). While a strong sonication cycle can easily kill gram-negative bacteria, gram positive ones withstand higher sonication rates [270]. Moreover, our results are in agreement with the study performed by Joyce *et al .*[273].

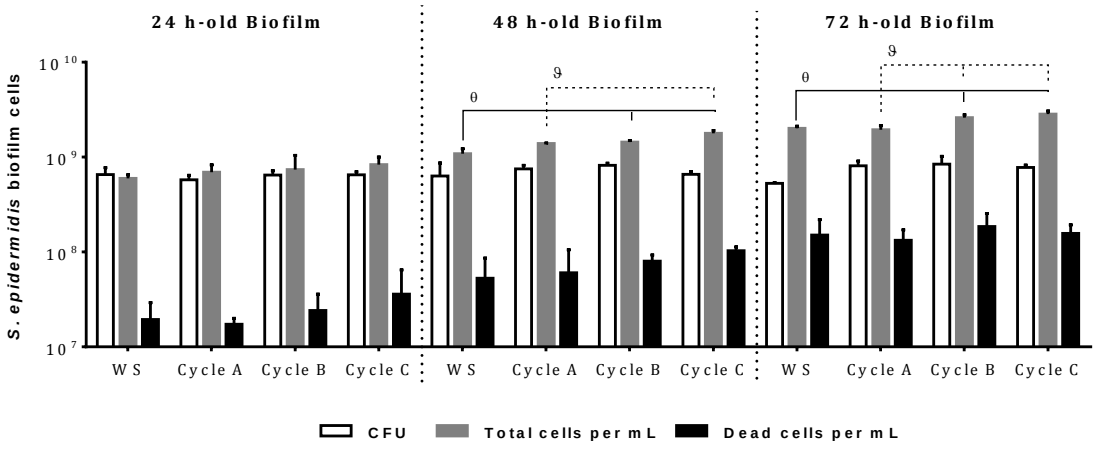

Figure 2.A.3: Quantitative results obtained by CFU counting and by fluorescence microscopy in 24 h-, 48 h-, and 72 h-old biofilms, after each sonication treatment. Bars represent the mean ± standard deviation of bacterial cell number within a biofilm sample after its treatment. Results are representative of three independent experiments. Statistical differences ($\rho < 0.05$) between the no-sonication control (WS) and any sonication cycle (θ) or between sonication cycle A and other sonication cycles (ϑ) were analyzed with ANOVA Tukey's test.

To determine if our observations could be extrapolated to other *S. epidermidis* strains, we selected two other *S. epidermidis* biofilm-forming strains (1457 and

RP62A) to validate our findings. These strains were selected since they show distinct ability to produce biofilm: 9142 and 1457 strains produce a denser and crusty biofilm, whereas RP62A produce a smoother biofilm [274].

As illustrated in the *table 2.A.1*, similar results were found in comparison to the strain 9142, validating the model strain used in this study. Of note, strains 9142 and 1457 had a significantly higher biomass than RP62A, although those differences were not correlated with the CFU count in 72 h-old biofilms. Therefore, this can easily be explained by the fact that a biofilm is the sum of bacteria and the matrix [24], suggesting that 1457 and 9142 strains may accumulate a denser surrounding matrix.

Table 2.A.1: Biomass quantification and viable and total enumeration cells from biofilms of *S. epidermidis* 9142, 1457 and RP62A strains grown for 24, 48 and 72 h. The values represent the mean ± standard deviation of 2 to 3 independent experiments. Statistical differences ($\rho < 0.05$) between OD (*), CFU (ψ) and total cells (θ) in the different strains were analyzed with ANOVA Tukey's test.

	24 h-old Biofilm	***48 h-old Biofilm***	***72 h-old Biofilm***
9142 strain			
OD_{595mn}	1.61±0.05*	4.02±0.03*	5.84±0.41
CFU[a]	6.48±0.68E+08	8.18±0.44E+08$^{\psi}$	8.43±1.76E+08
Total cells[a]	7.40±3.05E+08	1.44±0.06E+09	2.45±0.49E+09
1457 strain			
OD_{595mn}	1.92±0.07*	5.72±0.33*	6.79±0.23
CFU[a]	6.15±0.35E+08	6.11±0.12E+08$^{\psi}$	6.63±1.03E+08
Total cells[a]	8.13±0.30E+08	1.96±0.24E+09	3.37±0.53E+09
RP62A strain			
OD_{595mn}	0.41±0.01*	1.72±0.04*	2.30±0.03*
CFU[a]	1.97±0.12E+08$^{\psi}$	4.38±0.16E+08$^{\psi}$	4.45±0.07E+08
Total cells[a]	2.21±0.41E+08	5.84±0.95E+08$^{\theta}$	7.78±0.99E+08$^{\theta}$

In all the tested sonication conditions we were able to suspend the biofilm without visible cell clusters. By fluorescence microscopy, we found that while the size of cell clusters was reduced by increasing the sonication period, they were nonetheless, present (*Fig. 2.A.4A*). As microscopic counting is only feasible with a small sampling size, and therefore homogeneity of the suspension is crucial, we devised a simple experiment to determine the presence of the microscopic cell clusters. This experiment was based on the sedimentation velocity of particles of different sizes, as determined by OD readings. Since cell clusters would sediment

quicker than individual bacteria, this could be detected as a reduction of the OD_{595mn}. As illustrated in *figure 2.A.4B*, we observed a faster decrease on the OD measurements, mainly in mature biofilms, confirming our qualitative microscopic observations.

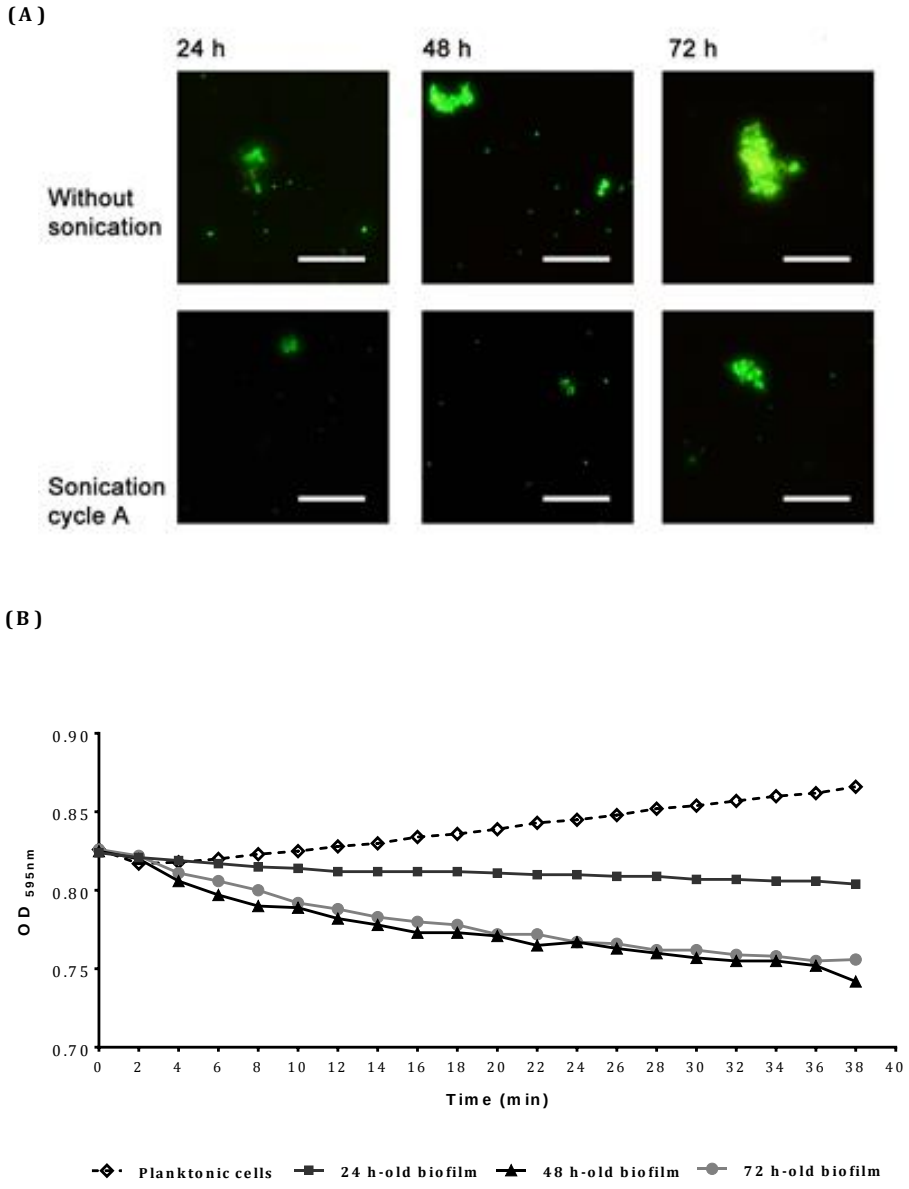

Figure 2.A.4: Effect of bacterial cell aggregates during the time course of biofilm formation: *(A)* Examples of cell clusters observed by fluorescence microscopy. **(B)** OD_{595nm} measurements over time. Results are representative examples of three independent experiments.

2.B. Optimization of a qPCR Gene Expression Quantification Assay

2.B.1. Material and Methods

Bacterial strain and growth conditions

The 9142 *S. epidermidis* strain previously characterized regarding biofilm formation capacity was used in this work [265]. The 24 h-old biofilms were formed as previously described on the *sub-chapter 2.A.1*. Biofilms were washed and resuspended in 1 mL of 0.9% NaCl. Planktonic bacteria were grown for 18 h in 2 mL TSB at 37 ºC in an orbital shaker at 120 rpm (10 mm orbit diameter, Biosan).

RNA extraction

For RNA extraction, two commercially available kits with distinct principles – FastRNA® Pro Blue (MPBiomedicals) and PureLink™ RNA Mini Kit (Invitrogen) – were selected and total RNA was isolated according to the manufacturers' instructions. A custom protocol based on the ISOLATE RNA Mini kit columns system (Bioline) was also optimized. Briefly, bacteria were first resuspended in 100 mL RNase free water and transferred to a 2 mL safe lock tube containing 0.4 grams of acid washed 150–212 mm silica beads (Sigma), 400 mL Lysis buffer R (provided by the kit) and 400 mL 90% phenol solution (AppliChem) were then added. This mixture was vortexed for 20 sec before using the FastPrep® cell disruptor (BIO 101, ThermoElectron Corporation, Thermo Scientific), with setting 6.5 for 35 sec. The beat-beading step was repeated twice and the samples were cooled on ice for 5 min between steps. Samples were centrifuged at 16,000 g for 5 min and supernatants transferred to a new tube and mixed with equal volume of 100% ethanol (Fisher Scientific). The samples were them transferred to the silica columns and centrifuged at 12,000 g for 15 sec. The flow-through was discarded and each column was reinserted into a new collection tube. 700 mL of Wash buffer I were added to each column and centrifuged at 12,000 g for 15 sec. The flow-through was discarded, the column was inserted into the same collection tube and 500 mL of Wash buffer II were added to each column, and centrifuged at the same conditions. The flow-through was discarded and the columns reinsert into a new

collection tube, for a new centrifugation. The collection tube was discarded and each column was inserted into a recovery tube. Finally, RNA elution was achieved by adding 45 mL of RNAse-free water, incubated for 1 min and then centrifuged for 1 min at 12,000 g. All steps were done at room temperature, except where otherwise noticed.

DNase I treatment and RNA quality determination

After RNA extraction, a DNase I (Fermentas) treatment step was included to digest remaining genomic DNA (gDNA). Briefly, 5 µL of reaction buffer and 2 µL of DNAse I were added to each sample of RNA and incubated for 30 min at 37 ºC. To inactivate the DNAse I endonuclease, 5 µL of 25 mM EDTA were added to the reaction mixture, which was then incubated at 65 ºC for 10 min.

The RNA quality and quantity were determined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm using the NanoDrop 1000™ Spectrophotometer (ThermoScientific). Electrophoresis was carried out in a horizontal 1.5% agarose gel, containing ethidium bromide. The gel was visualized under a Gel Doc™ 2000 system (Bio-Rad).

Complementary DNA (cDNA) synthesis

cDNA synthesis was performed using 4 different commercial kits: Super Script® VILO™ (Invitrogen), RevertAaid™ First Strand cDNA Synthesis kit (Fermentas), iScript™ cDNA synthesis (Bio-Rad) and qScript™ cDNA Synthesis (Quanta BioSciences). Briefly, total RNA was normalized to 500 ng, mixed with 10 pmol of each reverse primer to a final volume of 20 µL, incubated for 5 min at 65 ºC and chilled on ice. The normalized RNA was reverse transcribed in two final reaction volumes: 20 µL and 10 µL. The 20 µL volume reactions contained the following: 4 µL of 5x RT buffer, 10 mM dNPTs mix, 200 U of Reverse Transcriptase enzyme, 20 U of RiboLock RNAse inhibitor, half of the normalized RNA pre-mixture and nuclease free water up to 20 µL. The 10 µL reactions contained half the corresponding volumes. The cDNA mixture was then incubated for 60 min at 42 ºC followed by 10 min at 70 ºC. To determine the possibility of gDNA carry-over, control reactions were performed under the same conditions, but lacking the reverse transcriptase enzyme (no-RT control).

Quantitative real time PCR (qPCR)

The qPCR analysis was performed using 4 different commercial qPCR master mixes: mi-real-time EvaGreen® Master (Metabion), Maxima® SYBR Green Master Mix (Fermentas), iQ™ SYBR® Green Supermix (Bio-Rad) and PerfeCTa® SYBR® Green SuperMix (Quanta BioSciences). A 100-fold dilution of the resulting cDNA template was used for qPCR analysis of *icaA* gene-specific primers and both 20 and 10 µL reaction volumes were performed. The 20 µL reaction contained 2 µL diluted cDNA or no-RT control, 10 pmol of the primer (*16S*: FW: 5' – GGGCTACACACGTGCTACAA – 3' and RV: 5' – GTACAAGACCCGGGAACGTA – 3'; *icaA*; FW: 5' –TGCACTCAATGAGGGAATCA – 3' and RV: 5' – TAACTGCGCCTAATTTTGGATT – 3'), 6 µL nuclease free water, and 10 µL of the respective master mix. The 10 µL reactions contained half the corresponding volumes. qPCR run was performed on a CFX 96 (Bio-Rad) as follow: 95 ºC for 30 s, 39 cycles of 95 ºC for 5 sec, 60 ºC for 15 sec and 68 ºC for 15 sec. Relative fold increase of *icaA* in biofilms compared to the planktonic cultures was normalized to the expression of the internal control gene (16S). qPCR products were analyzed by melting curves for unspecific products or primer dimer formation. Relative fold increase of specific mRNA transcripts was calculated using 2DCt method, a variation of the Livak method, where 2 stands for the 100% reaction efficiency (the reaction efficiency was determined experimentally and thus 100% efficiency was replace by the real efficiency) and DCt =Ct (housekeeping gene)-Ct (target gene). The values of the PCR efficiency calculated to each master mix were: 84±4% for iQ™ SYBR® Green, 84±7% for Maxima® SYBR Green, 78±5% for PerfeCTa® SYBR® Green and 87±6% for mi-real time EvaGreen® master mix. The data analysis was based on at least three independent experiments.

Statistical analysis

The assays were compared using one-way analysis of variance (ANOVA) by Tukey's multiple comparisons test and the paired sample *t*-test, using SPSS. All tests were performed with a confidence level of 95%.

2.B.2. Results and Discussion

RNA extraction

The RNA extraction was first performed using the commercial FastRNA® Pro Blue kit (MPBiomedicals), which combines mechanical and chemical lysis together with organic extraction. We then repeated the process using the PureLink™ RNA Mini Kit (Invitrogen) that uses an enzymatic lysis and silica membrane extraction. In addition, a customized protocol based on both mechanical and chemical lysis, required to effectively extracting the RNA from *S. epidermidis* biofilms, with subsequent silica membrane isolation that will reduce the time needed for RNA extraction [275], was performed. This custom approach was tested with 4 different column systems as follow: ISOLATE RNA Mini kit columns system (Bioline), PureLink™ RNA Mini Kit (Invitrogen), Direct-zol™ RNA MiniPrep (Zymo Research) and FavorPrep™ Blood/Cultured Cell Total RNA (Favorgen).

The PureLink™ kit was the RNA extraction kit that yielded the poorer results (*Fig. 2.B.1)*. However, when combined the PureLink™ column system with the custom lysis we were able to improve the RNA yield up to similar values than those obtained with FastRNA® Pro Blue kit. All the other column systems tested resulted in high RNA yield. The absorbance ratio A260/A280 shows that all RNA extraction procedures resulted in acceptably low levels of protein contamination. Some of the kits presented an A260/A230 ratio below 1.8, indicating possible contamination with polysaccharide, phenol and/or chaotropic salts. Despite the importance of such parameters in RNA quality determination, they have trivial implications in the stability and reliability of gene expression assays [276].

Regarding the RNA quality and yield, none of the custom protocols exceed the FastRNA® Pro Blue kit. However, the reduction of costs and preparation time were also taken into account. As listed in *table 2.B.1*, with our custom RNA protocol we could achieve a 75% and 68% reduction of time and cost per reaction, respectively, in comparison with FastRNA® Pro Blue kit.

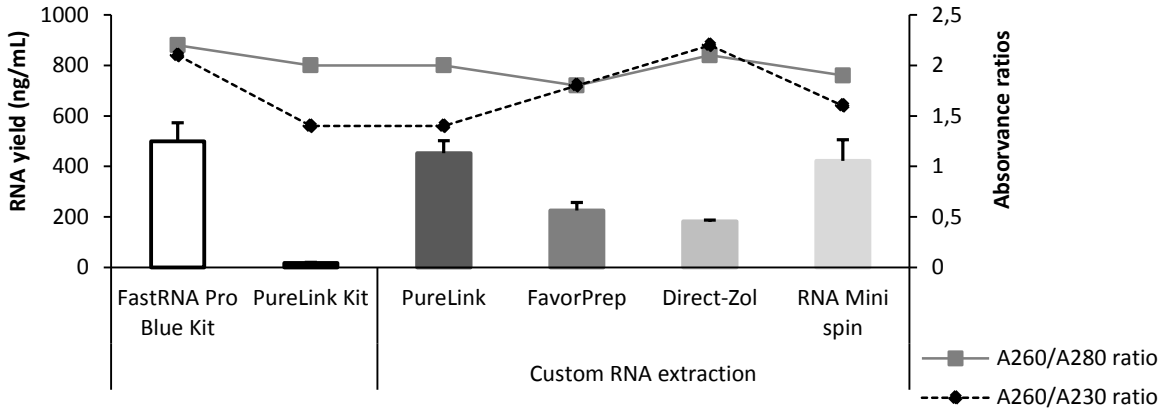

Figure 2.B.1: Comparison of RNA yield and purity obtained by the different RNA extraction procedures used. The values represent the mean ± the standard deviation of 3 independent experiments.

Table 2.B.1: Costs associated with kits and reagents used for the RNA extraction. All the prices listed were obtained by quote during January 2012.

Reagents and Kits (Manufacturer)	Number of reactions per kit	Prices (€) per reaction
FastRNA® Pro Blue (MPBiomedicals)	50	7.14
PureLink™ RNA Mini Kit (Invitrogen)	out-50	9.70-4.46
ISOLATE RNA Mini Kit (Bioline)	10-250	5.30-3.68
Direct-zol™ RNA MiniPrep (Zymo Research)	50-200	4.19-3.41
FavorPrep Blood/Cultured Cell Total RNA (Favorgen)	50-300	2.50-1,80
Ethanol 100% (Fisher)	2500*	0.006
Chloroform (Fisher)	3333-8333	0.002-0.001
Phenol (AppliChem)	277-1387	0.08-0.06
Glass beads, acid-washed, 150-212 mm (Sigma)	25-1250	1.53-0.33
RNAse & DNAse free tubes with screwcap (BioPlastics)	500	0.12

cDNA and qPCR reaction optimization

The performance of different reverse transcriptase kits and qPCR master mixes commercially available were tested. Using the cDNA synthesized by different kits, *icaA* gene expression was quantified by qPCR with distinct master mixes. Interestingly, significant differences in *icaA* mRNA levels were found when varying the reverse transcriptase kits ($\rho < 0.05$), but no changes were observed when different qPCR master mix are used ($\rho > 0.05$), excepted in the case of RevertAaid™ First Strand cDNA Synthesis kit as illustrated in *figure 2.B.2*.

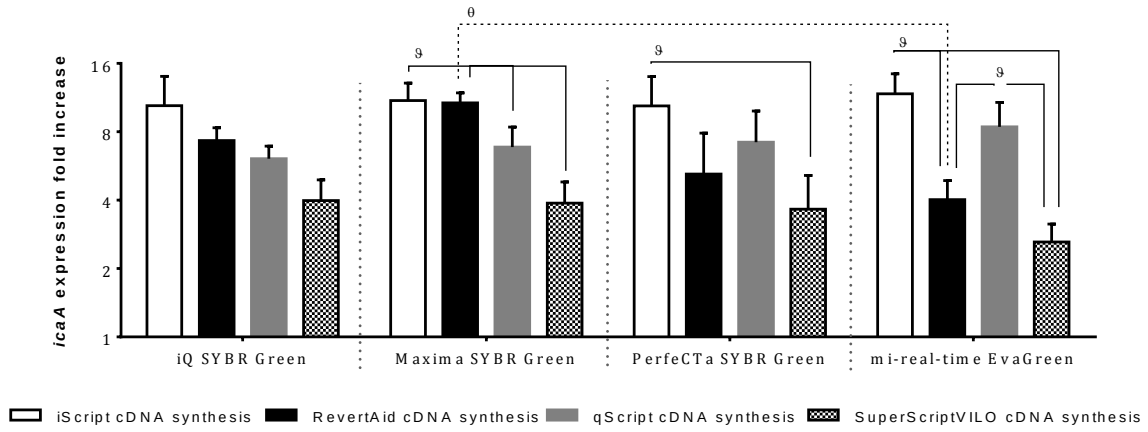

Figure 2.B.2: The impact of different cDNA and qPCR commercial kits in *icaA* gene expression quantification. The values represent the mean ± standard deviation of 3 independent experiments. Statistical differences ($p < 0.05$) between cDNA kits (ϑ) or qPCR master mixes (θ) were analyzed with ANOVA Tukey's test.

Reduction in the reverse transcriptase and qPCR volume reaction are among the possible ways to reduce costs associated with gene expression analysis. However, smaller volumes may introduce more pipetting errors. In order to determine if a reduction in cDNA reaction volume could impair the qPCR results, and *vice-versa*, the RNA extracted from both *S. epidermidis* planktonic and biofilm cultures were first reverse transcribed using the four cDNA synthesis kits with either 10 or 20 µL of volume and then quantified with 20 µL volume reaction of Maxima® SYBR Green Master Mix (*Fig. 2.B.3A*). Simultaneously, qPCR commercial kits were used with both 10 and 20 µL final reaction volumes, using as template the reverse transcribed RNA obtained from a 20 µL reaction with RevertAid™ First strand cDNA synthesis kit (*Fig. 2.B.3B*).

As observed in the *figure 2.B.3*, the variation of qPCR volume did not affect the quantification of *icaA* gene expression ($p > 0.05$). On the other hand, regardless the different cDNA kits used, a significant variation was found in the cDNA obtained using SuperScript® VILO™ cDNA.

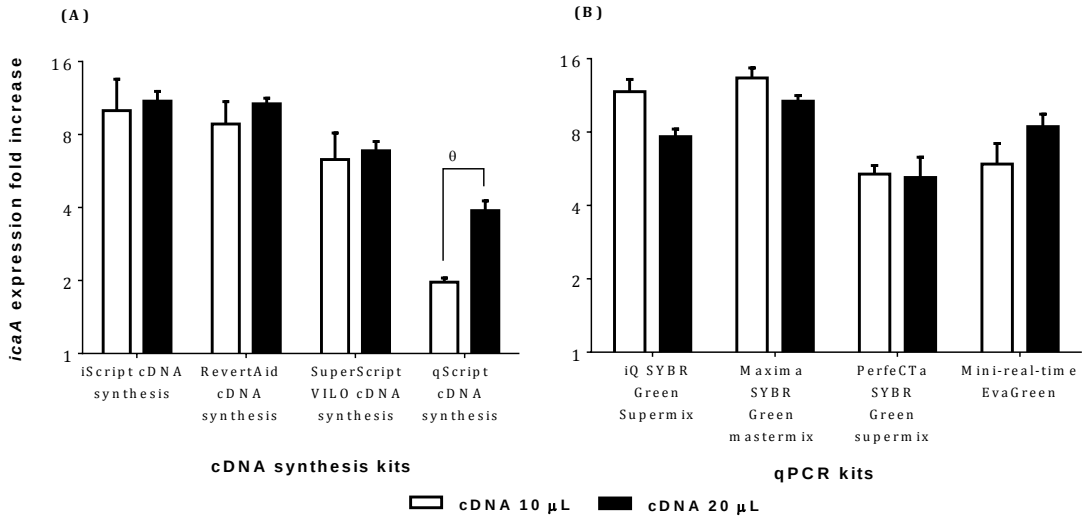

Figure 2.B.3: Variation in *icaA* gene expression quantification using different cDNA (A) or qPCR (B) reaction volumes. **(A)** cDNA synthesized using either 20 µL or 10 µL reaction volumes was used for *icaA* transcripts quantification. The transcripts were detected using Maxima® SYBR Green Master Mix. **(B)** cDNA synthesized using RevertAid™ First Strand cDNA synthesis kit (20 µL reaction) was used for *icaA* transcripts quantification by different qPCR master mixes and using 20 µL or 10 µL final reaction volumes. The values represent the mean ± standard deviation of 3 independent experiments. Statistical differences ($p < 0.05$) between 10 µL and 20 µL reactions (θ) were analyzed with paired t-test.

2.2. General conclusions

The *chapter 2* introduces some of the most used methods in clinical and research microbiology laboratories to assess bacterial quantification in catheter-related infections. The sensitivity of diagnostic assays has been improved in the last years through introduction of techniques that remove adherent biofilm bacteria from the medical implant [236, 269, 277]. Despite the new improvements, the diagnosis of these infections is still difficult. Molecular and image techniques have also been accepted as more sensitive tools for improving the diagnosis of these infections [242, 252, 272]. Moreover, both conventional culture and molecular methods can be complementary, but biofilm samples are still challenging. The surrounding matrix confers complexity to biofilm cells, hampering quantification methods by either promoting cells clusters or contributing to a source of genetic material contamination. Also, optimum conditions to effectively break and properly quantify the biofilm need to be optimized. In this particular case, biofilm and gene

expression quantification methods were adapted and optimized in order to reach optimal and feasible results when applied to *S. epidermidis in vitro* biofilms.

Establishment of clusters is a paramount issue in the usage of conventional methods to quantify cells from biofilms. To circumvent this issue, we started by testing the effect of different sonication conditions in young (24 h-old) or mature (48 and 72 h-old) biofilms and evaluated its effect in the quantification outcome using three distinct methods. Despite the limitations of CFU count and OD measurements, the addition of a sonication step promoted some technical improvements. We also tested an automatic image counting software, in association with fluorescence-based microscopy, in order to accurately and quickly quantify the number of *S. epidermidis* total and viable biofilm cells. While some further optimization is still needed, our semi-automatic counting has proven to be robust a method and brings important advantages [254, 266, 267]. The speed of processing is of upmost importance, as manual counting is time consuming. Furthermore, by applying the same settings, automatic counting is more reliable, since it does not depend on user to user interpretation variability.

We also addressed the issue of reliable gene expression within *S. epidermidis* biofilms. We demonstrated that it was possible to achieve a robust gene expression protocol by customizing some commercial available kits, with a substantial reduction of the economic cost of the experiment. We also showed that the reduction of either cDNA or qPCR volume reaction are among the possible ways to reduce costs associated with gene expression analysis without compromising the reproducibility and accuracy of the method.

CHAPTER 3

MOLECULAR AND PHENOTYPICAL CHARACTERIZATION OF *S. EPIDERMIDIS* ISOLATED IN A PORTUGUESE HOSPITAL

This chapter provides a general characterization of 86 Portuguese clinical isolates of *S. epidermidis* regarding the ability of each isolate to form biofilm and the carriage of biofilm-related genes. The antibiotic resistance and its association with clinical, phenotypic and molecular features were also pointed out.

3.1 Introduction

Owing to multidrug resistant organisms and financial impact to the communities, healthcare-associated infections (HAIs) represent today's a significant problem in healthcare facilities worldwide, impairing patient's quality of life [5, 6]. These infections are associated with a variety of risk factors that includes the catheter insertion site itself and surgical procedures [9, 12]. The length of catheterization and the overuse or improper use of antibiotics, are also associated to significant morbidity and mortality mostly among immunocompromised and critically ill patients or very young and very old people [1, 3, 7]. Furthermore, transmission of infectious diseases between healthcare workers and patients is also very common [7]. The risk increase, if the causative organism has developed resistance to a number of antimicrobial agents [5, 9]. Although a natural phenomenon [278], antibiotic resistance is accelerated by use of antimicrobial drugs and can eventually lead to failed treatments and/or deaths, as well as and growing healthcare costs [6, 278]. In fact, the emergence and spread of highly-resistant bacteria is now a major threat to public health and is widely recognized, by the three most important international health organizations, as a serious global problem [7, 278, 279]. HAIs, that usually occur two to three days after admission to hospital [280], account for 5 to 10% of all hospital admissions [5, 7]. The two most recent Portuguese national surveillance reports, concerning healthcare infections, account for an overall prevalence of 11.4% in 2009 [281] and 10.6% in 2012 [10], the highest among European countries [7].

A part of the human skin microbiota, the coagulase-negative bacterium *S. epidermidis* is now considered, an opportunistic pathogen responsible for many HAIs, mainly those related to indwelling medical devices (such as, prosthetic joints, cardiac pacemakers and mechanical heart valves, artificial lenses, central venous catheters cerebrospinal fluid shunts, among others) [1, 12, 282]. With an isolation rate of approximately 7% among all HAIs and the most frequently isolated microorganism in bloodstream infection [7], *S. epidermidis* is considered one of the most adaptable nosocomial human pathogens [11, 12]. Due to the capacity to accumulate multiple antibiotic resistance determinants by genetic recombination and gene acquisition [28], multidrug resistance (MDR) is increasing among Staphylococcal species leading to a major health concern not only in Portugal but in all industrialized countries [7, 278]. For instance, the *S. epidermidis* resistance to

methicillin/oxacillin (MRSE) encoded by *mecA* gene, currently exceeds 70% in many institutions worldwide [283-287]. In Portugal, a recent study revealed *mecA* prevalence, among Portuguese *S. epidermidis* isolates, to be 79.8% [155]. Antibiotic resistance is therefore accelerated by the use of antimicrobial drugs and can eventually lead to failed treatments and in some case, deaths [7, 278], as well as growing healthcare costs [6, 278]. Although antimicrobial resistance compromise therapy, biofilm formation enhances this problem by affecting the efficacy of the administrated treatment leading to persistent infections, compromising patient safety [26]. In the recent years, indwelling medical devices have been crucial in the improvement of patient care however this progress, in an inadvertently manner, also predisposed patients to biofilm-related infections [12, 26]. The ability to form a community of adherent microorganisms encased in a self-produced extracellular matrix (termed biofilm) is considered the major determinant of *S. epidermidis* virulence [23]. *S. epidermidis* strains are known to vary in their ability to form biofilms and several genes have been shown to take a part in this complex process [28, 288]. Among these, the most extensively studied are in the *icaADCB* operon (*ica*, intercellular cluster adhesin) which is responsible for the synthesis of polysaccharide intercellular adhesin (PIA), a major component of the biofilm matrix, directly impacting in the biofilm accumulation [289]. Besides *icaADBC* operon, the *aap* (accumulation-associated protein) [290] and *bhp* (biofilm-homologous *S. aureus* protein) [89] genes are also involved in and considered important genetic determinants of *S. epidermidis* biofilm formation in a PIA-independent manner. Additionally, *icaA*, *aap* and *bhp* genes are all involved in sessile biofilm cell proliferation and maturation [26, 88], thus being major contributors to biofilm development and persistence.

Despite the importance and clinical significance, there is a lack of available information relating epidemiology data to the molecular and phenotypic characteristics of *S. epidermidis* clinical isolates in Portugal, mainly related to biofilm formation and antimicrobial susceptibility.

Here, we determined the phenotypic and molecular characteristics of *S. epidermidis* isolates recovered from patients diagnosed with device-related infections, over a 30-month period. Their antibiotic resistance profile as well as its association with phenotypic and genotypic biofilm-associated determinants was also assessed. To the best of our knowledge this was the first study performed

with Portuguese clinical isolates that correlates the biofilm forming capacity to the antimicrobial resistance pattern. Importantly, although surveillance allow the determination of infection rate and the assessment of the effectiveness of interventions, the phenotypic and molecular characterization of clinical isolates may improve the management of those preventive strategies and be useful for a better understanding of the epidemiology of *S. epidermidis* associated infections.

3.2 Material and Methods

Isolation of clinical *S. epidermidis* isolates

A total of 86 *S. epidermidis* isolates were collected from January 2011 to June 2013 in the Microbiology Laboratory of the Hospital de Santo António, a 700-bed tertiary-care public hospital in Porto (Portugal) that handles about 35 000 inpatients admissions per year. All clinical isolates came from patients aged from newborns (≤ 1 year-old) to 94 year-old with a diagnosis of HAIs associated to device colonization, clinically and laboratorial confirmed, following Infectious Diseases Society of America guidelines [291]. Additionally, HAIs were defined as a localized or systemic condition resulting from an adverse reaction to the presence of an infectious agent(s) or its toxin(s) in accordance with CDC (Centers for Disease Control and Prevention) criteria [280]. That condition occurred 48 hours or more after hospital admission and was neither present nor incubating at the time of admission [280]. Patients clinical and demographics data were collected under approval of Ethics Committee Board of Hospital de Santo António, Porto Hospital Centre, Portugal (Reference 015/09: 014-DEFI/014-CES).

Identification of clinical *S. epidermidis* isolates

Each isolate was identified at the species level using the commercially available VITEK® 2 identification system using the gram-positive ID card (BioMérieux) and subsequently by matrix assisted laser desorption ionisation – time-of-flight (MALDI-TOF; BioMérieux), according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Susceptibility to penicillin, clindamycin, erythromycin, daptomycin, fusidic acid, fosfomycin, gentamicin, levofloxacin, moxifloxacin, linezolid, rifampin, tetracycline, tigecycline, vancomycin, teicoplanin and trimethoprim-sulfamethoxazole was determinate by VITEK® 2 using the P619 panel according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Results for each clinical isolate was reported using the category interpretations "susceptible", "intermediate" and "resistant". *S. epidermidis* isolates were considered as multidrug resistant (MDR), if non-susceptible to at least one agent in 3 or more antimicrobial categories according to standardized international terminology [292].

Quantification of biofilm formation

Biofilm cultures of each strain were performed in batch mode as previously described in *chapter 2* with minor modifications. Briefly, a starter culture was grown overnight in TSB (Liofilchem) at 37 °C with aeration and agitation (120 rpm, 10 mm orbit diameter, Biosan). Overnight cultures were adjusted to an OD at 640 nm of 0.25-0.3 corresponding to 2_x10^8 CFU per mL. Subsequently, 200 µL of 100-fold dilution performed in TSB supplemented with 0.4% (w/v) of glucose to induce biofilm formation, was placed in a 96-well plate (Orange Scientific) and incubated in the same conditions as the starter culture for 24 hours. Furthermore, quantitative determination of biofilm formation was performed as previously described by Stepanovic *et al.* [240] with some modifications. Briefly, after incubation time, the bacterial cells in suspension were carefully removed and each well was washed twice with 200 µL of 0.9% of NaCl. Afterwards, 100 µL of 99.9% methanol (Fisher Scientific) was added to each well and let it in for 15 min in order to fix the biofilm. Methanol was then removed and the plate was left to air dry. The fixed bacteria biofilm cells were stained with 200 µL of 1% (v/v) CV (Merck) per well, for 5 min. Excess crystal violet was removed by gently washing each well twice with distilled water and filled with 160 µL of 33% (v/v) glacial acetic acid (Fisher Scientific) in order to solubilize the CV. Absorbance was measured at 570 nm ($OD_{570\ nm}$) using a ELISA plate reader (Tecan). Additionally, 4 well per plate with sterile TSB alone were used to check for medium sterility and also served as negative controls in this assay. Sixteen replicates of each isolate per biofilm assay

were included and a minimum of three independent assays were carried out. Moreover, a biofilm producer strain (*S. epidermidis* RP62A) and a non-biofilm producer (*S. epidermidis* ATCC 12228) were also included. The optical density of each isolate was compared with the mean absorbance ($OD_{570\ nm}$ < 0.26) of the negative control (ATCC 12228) and the ordinal classification for the level of biofilm production proposed by Stepanovic *et al*. [240] was used in order to simplify the data.

Gene detection by PCR

One to five bacterial colonies of each isolate were inoculated from a TSA agar plate into 200 µL of nuclease-free water. The cells were lysed by heating at 95 °C for 10 min followed by 5 min on ice. Cellular debris was removed by centrifugation at maximum speed for 5 min. One µl of the collected supernatant was used as template for PCR amplification. For single target amplification, the PCR was performed in the MJ Mini thermal cycler (Bio-Rad) with a final volume of 10 µL of final volume and containing 5 µL of DyNAzyme II PCR Master Mix 2x (Finnenzymes), 1 µL of primer mixture with a 10 µM concentration each and 2 µL of nuclease-free water. The primers sequences of the virulence-associated genes used in this study are listed in *table 3.1*. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of DNA denaturation at 94 °C for 30 sec primer annealing at 56 °C for 30 sec, and primer extension at 72 °C for 45 sec. After the last cycle, a final extension step at 72 °C for 10 min was added. Total PCR products were analyzed by gel electrophoresis with 2% agarose (Bio-Rad) stained with Midori Green DNA stain (Nippon Genetics Europe GmbH) and visualized by GelDoc® 2000 (Bio-Rad). A 100-bp DNA ladder (NZYTech) was used as a marker. A mock PCR reaction lacking the DNA template was prepared and used as a negative PCR control. In addition, *S. epidermidis* RP62A was included as positive PCR control and the *rpoB* gene was used as an internal control in each PCR run. *S. epidermidis* isolates were considered to harbor any of the tested genes if having at least one positive PCR result.

Table 3.1: Oligonucleotide sequences used in PCR gene amplification.

Gene	Oligonucleotide sequence (5' to 3')	PCR product size (bp)
PCR amplification of methicillin-resistance gene		
mecA set 1	Fw: CCG AAA CAA TGT GGA ATT GG Rv: TCA CCT GTT TGA GGG TGG AT	600
mecA set 2	Fw: GGC CAA TAC AGG AAC AGC AT Rv: CTG CAA CGA TTG TGA CAC G	425
PCR amplification of biofilm-associated genes		
icaA set 1	Fw: TGC ACT CAA TGA GGG AAT CA Rv: TCA GGC ACT AAC ATC CAG CA	417
icaA set 2	Fw: TGC ACT CAA TGA GGG AAT CA Rv: TAA CTG CGC CTA ATT TTG GAT T	132
aap set 1	Fw: GCT CTC ATA ACG CCA CTT GC Rv: GGA CAG CCA CCT GGT ACA AC	617
aap set 2	Fw: GCA CCA GCT GTT GTT GTA CC Rv: GCA TGC CTG CTG ATA GTT CA	199
bhp set 1	Fw: TGG ACT CGT AGC TTC GTC CT Rv: TCT GCA GAT ACC CAG ACA ACC	213
bhp set 2	Fw: CGT TCC CTT GAT TGA GGT GT Rv: GTT ACG TGA ACG GGT CGA TT	404

Legend: bp, base pair; Fw, forward; Rv, reverse.

Statistical analysis

Comparison of categorical variables was performed using Fisher´s exact test and Pearson´s chi-squared test (χ^2) using SPSS Statistical software version 17.0 (SPSS Inc.). The Fisher´s exact test was used to test for association and the χ^2 was used to analyze the quantitative variables. Level of significance was set at *p*-value ≤ 0.05 and all tests were two-tailed.

3.3 Results

Study population and clinical characteristics

Over a 30-month period, 86 isolates of *S. epidermidis* were collected from patients with a diagnosis of device-related infection and treated in a tertiary-care hospital in Portugal's second major city (Porto). The rate of HAIs in this hospital during the time course of this study is described in the *table 3.2*. The prevalence rate was in

average 10.1%. Overall, urinary tract and respiratory infections were the most prevalent ones.

Table 3.2: Time-trend analysis of HAIs rate: clinical prevalence *versus* laboratory-confirmed.

	Hospital Parameters (%)		
Prevalence rate[a]	***2011***	***2012***	***2013[b]***
Total	10.7 / 9.4	9.7 / 9.4	11.2
Urinary tract infection	2.2 / 2.6	2.4 / 3.1	2.8
Bloodstream infection	1.5 / 0.8	1.2 / 2.4	1.1
Respiratory tract infection	3.7 / 3.1	2.5 / 0.8	3.9.
Surgical infection	2.2 / 1.1	0.8 / 1.6	1.7
Other	1.1 / 1.8	2.8 / 1.5	1.7
Laboratory based clinically validated incidence rate			
Total infections per 1000 day inpatient	4.7	4.2	4.9
Total	2.1	2.0	2.5
Urinary tract infection rate	1.0	1.0	1.4
Bloodstream infection rate	0.3	0.3	0.3
Respiratory tract infection rate	0.5	0.4	0.5
Surgical infection rate	0.2	0.2	0.2
Others	0.1	0.1	0.4

[a] The prevalence rate is evaluated two times a year (1st semester / 2nd semester)

[b] Values correspond only to the first semester of 2013

Of the 86 studied patients, 49 (57.0%) were men and the mean age was 46 years (95% CI, 39.1 – 53.2%) range from newborns to 94 years old *(Table 3.3)*. The distribution of infections in this public teaching hospital was 24.4% in newborns (≤ 1 year, n = 21) and major (39.5%, n = 34) in elderly patients with 65 or more years, following the same tendency of the overall healthcare facilities in Portugal [10]. Furthermore, at least 33.7% (n = 29) of our study population were immunocompromised patients. The most common co-existing conditions were cancer (16.3%, n = 14), dialysis (9.3%, n = 8) and chronic infections (3.5%, n = 3). Sepsis was diagnosed in 34.9% (n = 30) of the patients. Twenty-eight patients (32.6%) didn't receive any antibiotic therapy while from 10 patients (11.6%) no information was available prior to identification of the causative bacterium. Preceding *S. epidermidis* infection recovery, the number of patients receiving at least one antimicrobial agent increased up to 84.9% (n = 73) with major frequency in elderly patients (39.7%, in which 48.3% of these were under combined therapy). The most prescribed drug was vancomycin (53.5%), followed by amikacin (11.6%)

and imipenem (10.5%). Six patients (7.0%) were still without any specific antibiotic therapy after *S. epidermidis* isolation while from 7 patients (8.1%) we had no available information.

Table 3.3: Clinical parameters of the 86 patients diagnosed with device-related infections.

Patients Clinical Parameters	
Demographic characteristics	
Mean age (years)	46
≤1 year (% of patients)	24.4
>65 years (% of patients)	39.5
Male gender (% of patients)	57.0
Average length of hospitalization from admission to *S. epidermidis* isolation (no. of Days)	25
Underlying condition (% of patients)	
Immunocompromised (total)	33.7
Cancer	16.3
Hemodialysis	9.3
Chronic infections	3.5
Under antibiotic therapy (% of patients)	
Pre-culture	55.8
Post culture	84.9
Source (% of isolates)	
Blood	84.9
CVC	10.5
Other	4.7

Regarding the type of healthcare-associated infections (*Fig. 3.1*), central line-associated bloodstream infections (CLABIs) were by far the most frequently reported during the period under study, accounting for 60.5% (n = 52) of all clinical infections, while catheter-associated urinary tract infections (CAUTIs) and respiratory tract infections respectively accounted for 18.6% (n = 16) and 12.8% (n = 11). Furthermore, CLABIs were similarly distributed among the different age groups rather than CAUTIs that were more prevalent among elders (68.8%). Surgical-site infections (5.8%, n = 5) and skin and soft tissue infections (2.3%, n = 2) were less prevalent infections in our population of study. In addition, there was no association ($p > 0.05$) between age, sex and clinical characteristics and/or incidence of *S. epidermidis* infections.

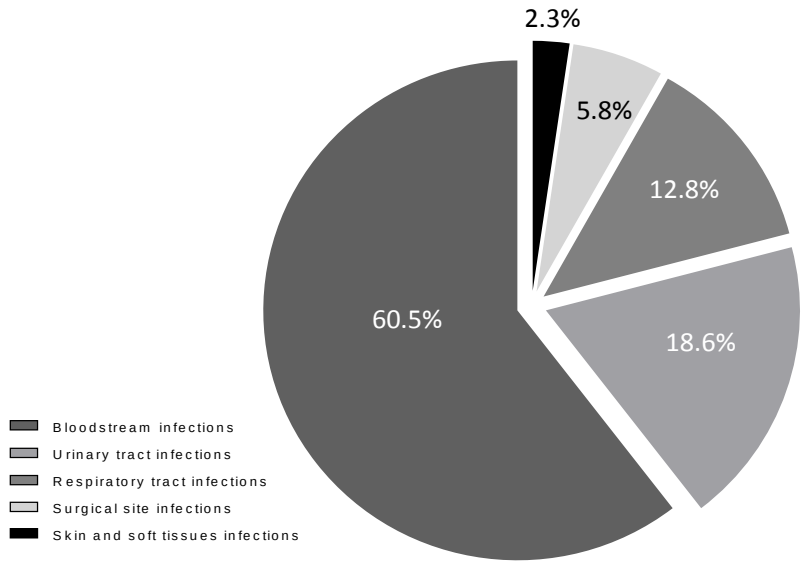

Figure 3.1: Type of HAIs related to the 86 *S. epidermidis* clinical isolates included in this study.

Antimicrobial susceptibility characteristics

Seventy-five (87.2%) *S. epidermidis* clinical isolates harbored *mecA*. The *mecA gene* encodes the altered penicillin-binding protein 2a and is associated with the methicillin resistance (MRSE) pattern [293]. Moreover, *S. epidermidis* isolates lacking *mecA* were considered as methicillin-susceptible *S. epidermidis* (MSSE). The analysis of antimicrobial susceptibility profiles (*Table 3.4*) has shown higher levels of resistance to β-lactam antibiotics (penicillin; 96.3%, n = 82), erythromycin (79.1%, n = 68), gentamicin (69.8%, n = 60) and tetracyclin (69.8%, n = 60). All *S. epidermidis* clinical isolates were susceptible to vancomycin, daptomycin and tigecycline. Linezolid (96.5%, n = 82) and fosfomycin (94.2%, n = 81) also demonstrated high levels of susceptibility. Seventy-two (83.7%) clinical isolates showed a high rate of susceptibility to teicoplanin whereas the others 14 (16.3%) isolates demonstrated an intermediary phenotype.

Additionally, the levels of non-susceptible to at least one agent in 3 or more antimicrobial categories hence considered as MDR, were unexpectedly high, affecting 86.0% of *S. epidermidis* clinical isolates included in this study. The MDR phenotype (p = 0.001) was observed among 68 MRSE (91.9%) and 6 MSSE (8.1%) clinical isolates. Interestingly, sixty-three (86.3%) patients, receiving at least one antimicrobial agent, were infected by MDR isolates suggesting that susceptibility profile of the isolated strain should be considered for therapy. A

positive association (p = 0.035) was also found among patients under antibiotic therapeutic and MRSE isolates (90.4%, n = 66). Furthermore, the MDR phenotype was equally distributed among the different age groups and when combining the results, no association (p > 0.05) was found between the MDR phenotype and the underlying medical condition.

Table 3.4: Antimicrobial susceptibility profile of *S. epidermidis* isolates included in this study.

Antibiotics	Non-susceptible isolates (%)
Penicillin (n =82)	96.3
Erythromycin (n = 86)	79.1
Fusidic acid (n = 86)	75.5
Levofloxacin (n = 86)	70.9
Gentamicin (n = 86)	69.8
Tetracycline (n = 86)	69.8
Clindamycin (n = 86)	58.1
Trimethoprim-sulfamethoxazole (n = 85)	42.4
Moxifloxacin (n = 86)	32.6
Rifampin (n = 86)	31.5
Teicoplanin (n = 86)	16.3
Fosfomycin (n = 86)	5.8
Linezolid (n = 85)	3.5
Tigecycline (n = 86)	0.0
Vancomycin (n = 86)	0.0
Daptomycin (n = 67)	0.0
Multidrug resistance (n = 86)	86.0

Phenotypic and virulence-associated genetic traits

Biofilm formation is a common phenotypic feature of *S. epidermidis.* In order to assess the ability to form biofilm of each clinical isolate, a biofilm-forming strain (RP62A; $OD_{570\ nm}$ = 1.08) and a non-biofilm producer (ATCC 12228) were used as positive and negative controls, respectively. *S. epidermidis* solates with an optical density minor than or equal to *S. epidermidis* ATCC 12228 ($OD_{570\ nm}$ < 0.26) were considered non-biofilm producers (NP). *S. epidermidis* isolates were divided into four groups depending on whether they produce a strong and fully establish biofilm (SP), a moderate or weak biofilm (MP and WP, respectively) or did not produce any biofilm (non-biofilm producer; NP). Regarding the biofilm-forming capacity *(Fig. 3.2)*, 61 (70.9%) clinical isolates were considered biofilm producers with the

following distribution: 9 (10.5%) isolates were strong producers, 11 (12.8%) were moderate producers and 41 (47.7%) were weak biofilm producers. Twenty-five (29.1%) isolates were considered non-biofilm producers and were equally distributed among the different age groups ($p > 0.05$).

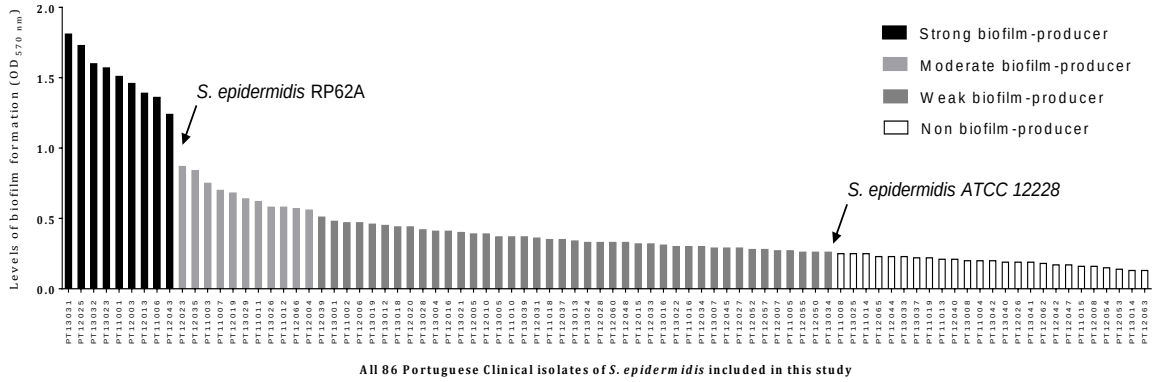

Figure 3.2: Distribution of biofilm formation capacity among the 86 Portuguese clinical isolates of *S. epidermidis* included in this study.

S. epidermidis clinical isolates with a stronger biofilm-producing phenotype were more prevalent among patients older than 1 year old (88.8%, n = 8). No significant differences ($p > 0.05$) in biofilm formation were observed between the MRSE and MSSE isolates, indicating no significant correlation between methicillin/oxacillin susceptibility and the ability to form *in vitro* biofilms. When comparing the antimicrobial resistance to the ability to form biofilm *(Table 3.5)*, fifty-three (71.6%) isolates with capacity to form biofilm appear to have a MDR phenotype ($p > 0.05$). Additionally, no association ($p > 0.05$) was found between MSSE and MRSE biofilm-forming isolates and MDR phenotype. Nevertheless, a strong association ($p = 0.007$) was found between MDR phenotype and the carriage of *mecA* gene. Interestingly, all (3.7%, n = 3) isolates susceptible to penicillin were also strong or moderated biofilm producers ($p = 0.013$) indicating that although showing great propensity to *in vitro* form biofilms, they were susceptible to one of the most used antibiotics worldwide. On the other side, the 3 isolates (3.5%) resistant to linezolid were poor biofilm formers ($p > 0.05$). Moreover, positive associations were also found between biofilm formation and some antibiotic susceptibility profiles such as, fusidic acid ($p = 0.033$) and gentamicin ($p = 0.001$).

Table 3.5: Relationship between antimicrobial non-susceptible profile and biofilm formation among *S. epidermidis* clinical isolates included in this study.

Antibiotics	Biofilm phenotype (%)			
	SP (10.5%, n = 9)	MP (12.8%, n = 11)	WP (47.7%, n = 41)	NP (29.1%, n = 25)
Penicillin (n = 82)	77.8	81.8	97.6	92.0
Erythromycin (n = 86)	66.7	81.8	78.0	84.0
Fusidic acid (n = 86)	44.4	81.8	75.6	84.0
Levofloxacin (n = 86)	44.4	54.5	78.0	76.0
Gentamicin (n = 86)	11.1	72.7	78.0	76.0
Tetracycline (n = 86)	66.7	100.0	61.0	72.0
Clindamycin (n = 86)	33.3	63.6	58.5	64.0
Trimethoprim-sulfamethoxazole (n = 85)	44.4	54.5	39.0	40.0
Moxifloxacin (n = 86)	33.3	45.5	36.6	20.0
Rifampin (n = 86)	44.0	45.0	29.0	24.0
Teicoplanin (n = 86)	0.0	9.1	17.1	24.0
Fosfomycin (n = 86)	0.0	0.0	12.2	0.0
Linezolid (n = 85)	0.0	0.0	4.9	4.0
Tigecycline (n = 86)	0.0	0.0	0.0	0.0
Vancomycin (n = 86)	0.0	0.0	0.0	0.0
Daptomycin (n = 67)	0.0	0.0	0.0	0.0
Multidrug resistance (n = 86)	66.7	100.0	87.8	84.0

Abbreviations: NP, non-biofilm producer; WP, week biofilm producer; MP; moderate biofilm producer; SP, strong biofilm producer.

Furthermore, the relationship between biofilm formation and the carriage of *icaA*, *aap* and *bhp* biofilm-mediating genes was assessed *(Fig. 3.3)*. Two sets of primers for each biofilm-associated gene were used, in order to minimize PCR amplification bias and false-negative results. The molecular determination of *icaA*, *aap* and *bhp* biofilm-mediating genes revealed that *aap* was the most prevalent gene, detected in 90.7% (n = 78) of the isolates, followed by *icaA* (64.0%, n = 55) and *bhp* (44.2%, n = 38). Regarding the association to biofilm phenotype, *icaA* and *aap* were more prevalent genes among isolates with strong and/or moderate biofilm-forming capacity, although *aap* seems more related to strong biofilm formation if in combination with the *ica* operon. Of interest, none of the clinical isolates was characterized by the presence of just *bhp* virulence-associated gene. Fifty-one out of 55 (92.7%) isolates were both *icaA*- and *mecA*-positive (p = 0.041). Also, 70 out of 78 (89.7%) *aap*-positive and 37 out of 38 (97.4%) *bhp*-positive isolates also carriage the *mecA* gene (p = 0.028 and p = 0.012, respectively). Additionally, the *icaA* gene was more common among MRSE biofilm

producers isolates (p = 0.006) while *aap* and *bhp* was rather more common among MRSE isolates with a weak or no *in vitro* biofilm-forming capacity ($p > 0.05$). Interestingly, the majority of the *S. epidermidis* isolates (83.7%, n = 72) were positive for at least 2 out of the 3 studied genes while only 4 (4.7%) isolates didn't carried any gene and demonstrated a weak biofilm formation capacity. Moreover, the genetic combination most frequently observed was *icaA*$^{+}$*aap*$^{+}$ (39.5%, n = 34), followed by *aap*$^{+}$*bhp*$^{+}$ (22.1%, n = 19) and by the carriage of the 3-gene combination (*icaA*$^{+}$*aap*$^{+}$*bhp*$^{+}$, 19.8%, n = 17). All clinical isolates that carriage the genetic combination *icaA*$^{+}$*aap*$^{+}$*bhp*$^{+}$ (n = 17) were associated with both MRSE (p = 0.001) and MDR phenotype (p = 0.001). Also, the majority of the isolates with the genetic combination *aap*$^{+}$*bhp*$^{+}$ demonstrated a methicillin-resistant and a MDR phenotype.

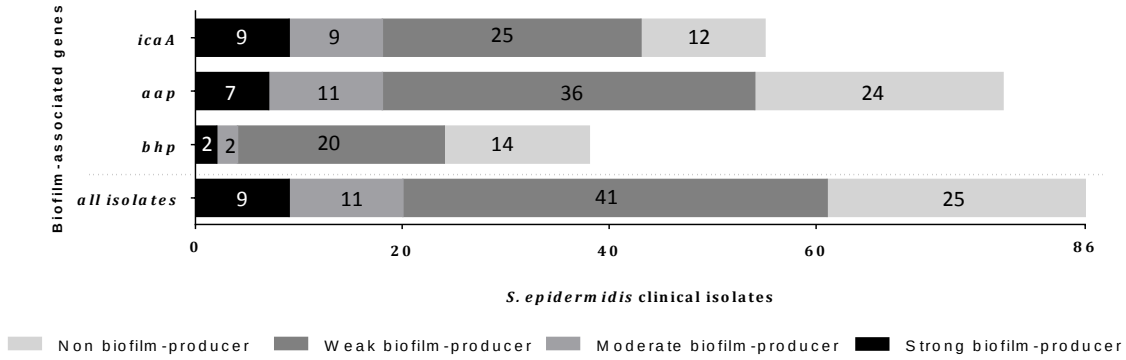

Figure 3.3: Relationship between biofilm-associated genes and biofilm phenotype among *S. epidermidis* clinical isolates included in this study.

3.4 Discussion

Despite the highly prevalence of HAIs in Portugal (10.6 % in 2012) [10], limited information is available regarding those causing by coagulase-negative Staphylococci and biofilm-forming *S. epidermidis* in particular. In addition, Portugal has one of the highest rates of antimicrobial resistance and antibiotic consumption across the European Union [7]. In the present study, we isolated 86 *S. epidermidis* clinical isolates and compared their phenotypic and molecular features with clinical data. The isolates were recovered from patients diagnosed with healthcare-associated infections and receiving treatment at a tertiary-care teaching

Portuguese hospital with an average rate of HAIs incidence of 10.1%, during the time course of this study. To the best of our knowledge, this is the first study addressing biofilm formation and *S. epidermidis* antimicrobial resistance of clinical isolates in Portugal. A summary table (*Table S3.A*) concerning all the information of the 86 studied isolates is present at the end of this chapter.

Bloodstream infections that included primary infections (*i.e.*, those not related to a secondary site) and secondary infections (*i.e.*, bacteremia associated with infection at another site) [280], were the most common type of infection among our study population which was not surprising, since *S. epidermidis* is the leading cause of bloodstream of infection episodes [7, 10, 11]. The prevalence of patients receiving at least one antimicrobial agent was unexpectedly high (84.9%) comparatively to other countries in European Union (32.7% in overall facilities) [7] and even to the reported national average (45.4%) [10]. According to the 2013 ECDC survey [7], the prevalence of antimicrobial use varied significantly among hospital types, the highest being observed in tertiary hospitals, which in part can explain the difference between the national (45.4%) and our local (83.7%) prevalence. It must be highlighted that the majority of these patients were already taking antibiotics for previously diagnosed infections. Additionally, these patients had several morbid conditions that, together with their age, suggest an immunocompromised status. Vancomycin alone or in combination, was the drug of choice, as recommended as a first line treatment for infections caused by methicillin-resistant Staphylococcal species [178].

The rate of *S. epidermidis* isolates resistant to methicillin was high (87.2%), as compared to similar studies [285, 294, 295]. Nevertheless, very high *mecA* prevalence has also been reported elsewhere [80, 286, 287, 296]. Additionally, a molecular epidemiologic study conducted in the Portuguese capital revealed an MRSE rate similar to those obtained in our local tertiary-care hospital (79.8% versus 85.4%, respectively) [155], suggesting a spread transmission of methicillin-resistant *S. epidermidis* across major healthcare facilities of Portugal. This also suggests that this spread of resistance by resistance genes accumulation *ie*, *mecA* gene carriage, is mainly due to the selective pressure exerted by the use of antibiotics. Indeed, *S. epidermidis* in particular MRSE, isolates are considered reservoirs of, antimicrobial resistance genes and prone to accumulates these genes [297], which is a cause of major concern because of associated higher risk of therapeutic failure.

Importantly, and similarly to previous reported observations by Hellmark, *et al*. [295], the majority of *S. epidermidis* clinical isolates included in this study demonstrated resistance to at least 3 classes of antimicrobial agents groups tested, hence multidrug resistant. The MDR phenotype was statistically correlated (p = 0.001) to MRSE clinical isolates, suggesting that resistance might be due to either accumulation of resistance genes and to high antibiotic pressure associated with hospital care [278]. Despite the high levels of resistance found in this hospital, our results are the expected for a device-related infection caused by *S. epidermidis*, as compared to other medical institutions.

Furthermore, antimicrobial resistance is an innate feature of Staphylococcal biofilms that in addition, to the increasing rates of reported antimicrobial resistance amongst clinical isolates impairs patient treatment [278]. As propensity for biofilm formation has an enormous clinical impact on drug resistance [34, 298], the *in vitro* biofilm forming capacity and the carriage of *icaA*, *aap* and *bhp* biofilm-associated genes of each clinical isolate was investigated. Similar to previously published studies [58, 93, 296, 299] the majority (70.9%) of our bacterial population exhibited *in vitro* biofilm-forming capacity, even if at different levels. The *aap* gene was the most commonly detected. However, *S. epidermidis* isolates that carried *aap* alone, demonstrated a diminished potential to *in vitro* form biofilms. These results indicated that this capacity may be enhanced by the presence of *icaADBC* genes although variable, as proposed by Stevens *et al.* [93]. In addition, we found a positive association (p = 0.023) between biofilm phenotype and the presence of *icaA* biofilm-associated gene, a result consistent with those obtained by Hira *et al.* [80]. Taken together our results confirm that *icaA* is a key part of the biofilm process and owns major relevance in the pathogenesis of *S. epidermidis* as in fact demonstrated by many other studies [80, 93, 286]. Regarding the role of *bhp*, we could not confirm any link to biofilm accumulation, also in accordance to other observation [58].

Interestingly, the observed phenotypic features of these clinical isolates are in agreement with the data reported in a recent Portuguese study performed with 61 community-acquired *S. epidermidis* isolates collected from healthy individuals from the same geographic region addressed in this study [300]. These findings raise some concerns as they demonstrate that *S. epidermidis* isolates recovered from the Portuguese community share a similar antibiotic resistance pattern and biofilm

formation ability to those recovered from the clinical setting. Together, these results point out that adequate antibiotic prescription is a growing necessity in order to minimize the antimicrobial resistance and clinical impact of *S. epidermidis* outside or inside of the healthcare institutions. Nevertheless, it should me mentioned that in both studies a limited number of isolates was studied. In the future, this limitation should be addressed to reach a more comprehensive understanding of healthcare infections associated to *S. epidermidis*, in Portugal.

Table S3.A: Phenotypic and molecular features of the 86 *S. epidermidis* isolates recovered from patients with a diagnosis of device-related infection.

	Phenotypic and molecular information			
Isolates	**Biofilm-forming capacity**	**Biofilm-mediating genes**	***mecA* detection**	**Non-susceptible profile**
PT11001	SP	*icaA+; aap+; bhp-*	+	PEN; FA; RIF;
PT11002	WP	*icaA-; aap-; bhp-*	nd	PEN; CLI; ERY; FA; GEN; LVX; SXT;
PT11003	MP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; LVX; MXF; RIF; TET; SXT;
PT11004	NP	*icaA+; aap+; bhp-*	+	PEN; ERY; RIF; TET; SXT;
PT11005	WP	*icaA-; aap+; bhp-*	nd	PEN; ERY; FOF; SXT;
PT11006	SP	*icaA+; aap-; bhp-*	+	PEN; LVX; TET; SXT;
PT11007	MP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; TET;
PT11008	NP	*icaA+; aap+; bhp-*	+	PEN; CLI; FA; GEN; LVX; MXF; TET; SXT;
PT11010	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; MXF; RIF; TET;
PT11011	MP	*icaA+; aap+; bhp+*	+	CLI; ERY; FA; GEN; TET;
PT11012	MP	*icaA-; aap+; bhp+*	+	PEN; FA; GEN; LVX; TET; TEC;
PT11013	NP	*icaA+; aap+; bhp-*	+	PEN; CLI; FA; GEN; LVX; MXF; LZD; TET; TEC; SXT;
PT11014	NP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT11015	NP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT11016	WP	*icaA+; aap-; bhp+*	+	PEN; FA; GEN; LVX; RIF;
PT11018	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; LVX; MXF; RIF; TET; SXT;
PT11019	NP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT12003	SP	*icaA+; aap-; bhp+*	+	PEN; RIF;
PT12004	MP	*icaA-; aap+; bhp-*	nd	PEN; ERY; FA; RIF; TET; SXT;
PT12005	WP	*icaA-; aap+; bhp-*	+	PEN; FA; LVX; MXF;
PT12007	WP	*icaA+; aap+; bhp-*	+	PEN; ERY;
PT12008	NP	*icaA+; aap+; bhp-*	nd	PEN; ERY; FA; GEN; TET;
PT12009	WP	*icaA+; aap+; bhp+*	+	PEN; FA; GEN; LVX; MXF; TET;
PT12010	WP	*icaA-; aap+; bhp+*	+	PEN; FA; GEN; LVX; SXT;
PT12013	SP	*icaA+; aap+; bhp-*	+	PEN; ERY; TET; SXT;
PT12015	WP	*icaA-; aap+; bhp+*	+	PEN; ERY; FA; GEN; LVX; MXF; TET; TEC; SXT;

PT12016	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; LVX; MXF; LZD; TET; TEC; SXT;
PT12019	MP	*icaA+; aap+; bhp-*	+	CLI; ERY; FA; GEN; LVX; MXF; RIF; TET; SXT;
PT12020	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; LVX;
PT12023	MP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; LVX; MXF; RIF; TET;
PT12025	SP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; RIF; TET;
PT12026	NP	*icaA-; aap+; bhp-*	nd	PEN; CLI; ERY; LVX; MXF; RIF; TET;
PT12027	WP	*icaA-; aap-; bhp-*	+	PEN; FA; RIF;
PT12028	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; RIF;
PT12031	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX;
PT12033	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX;
PT12034	WP	*icaA+; aap+; bhp+*	+	PEN; ERY; FA; GEN; LVX; RIF; TEC; SXT;
PT12035	MP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; TET; SXT;
PT12037	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET; SXT;
PT12039	WP	*icaA+; aap+; bhp-*	+	CLI; ERY; FA; FOF; GEN; LXV; MXF; TET; SXT;
PT12040	NP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; SXT;
PT12042	NP	*icaA-; aap+; bhp-*	+	PEN; ERY; FA; RIF; TET;
PT12043	SP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; LVX; MXF; RIF; TET;
PT12044	NP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; SXT;
PT12045	WP	*icaA-; aap-; bhp-*	+	PEN; FA; FOF; GEN; TET;
PT12047	NP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT12048	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT12050	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; LVX; MXF; RIF; TET;
PT12052	WP	*icaA-; aap+; bhp-*	nd	PEN; GEN; TET;
PT12053	NP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT12054	NP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET; TEC;
PT12055	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; LVX; MXF; RIF; TET; SXT;
PT12057	WP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX;
PT12060	WP	*icaA-; aap+; bhp+*	nd	PEN; ERY;
PT12062	NP	*icaA+; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; SXT;
PT12063	NP	*icaA+; aap-; bhp-*	nd	PEN; ERY; FA; SXT;
PT12065	NP	*icaA-; aap+; bhp+*	+	PEN; LVX; TEC;

PT12066	MP	*icaA+; aap+; bhp-*	nd	PEN; FA; GEN; LVX; MXF; RIF; TET;
PT13001	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; GEN; LVX; TET;
PT13004	WP	*icaA+; aap+; bhp-*	+	PEN; GEN; LXV; MXF; TEC; SXT;
PT13005	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; FOF; GEN; LXV; MXF; TET;
PT13007	WP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET; TEC;
PT13008	NP	*icaA-; aap+; bhp+*	+	PEN; ERY; FA; GEN; LVX; TEC;
PT13012	WP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET; TEC;
PT13013	WP	*icaA+; aap+; bhp-*	+	CLI; ERY; GEN; LVX; MXF; TET;
PT13014	NP	*icaA-; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; TET;
PT13016	WP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET; SXT;
PT13018	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; FOF; GEN; LXV; MXF; LZD; RIF; TET; TEC;
PT13019	WP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; TET; SXT;
PT13021	WP	*icaA-; aap-; bhp-*	nd	PEN; ERY; FA; GEN; TET;
PT13022	WP	*icaA-; aap+; bhp+*	+	PEN
PT13023	SP	*icaA+; aap+; bhp-*	+	PEN; ERY; FA; LVX; MXF; TET; SXT;
PT13024	WP	*icaA-; aap+; bhp+*	+	PEN; FA; GEN; LVX; TET; SXT;
PT13025	NP	*icaA+; aap+; bhp-*	+	CLI; ERY; GEN; LVX; MXF; TET; TEC; SXT;
PT13026	MP	*icaA+; aap+; bhp-*	+	PEN; ERY; LVX; MXF; TET; SXT;
PT13028	WP	*icaA+; aap+; bhp-*	+	PEN; ERY; LVX; MXF; TET; SXT;
PT13029	MP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; GEN; TET; SXT;
PT13031	SP	*icaA+; aap+; bhp-*	nd	PEN; ERY;
PT13032	SP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; FA; LVX; MXF; TET; SXT;
PT13033	NP	*icaA-; aap+; bhp+*	+	PEN; ERY; FA; GEN; LVX; RIF; TET; SXT;
PT13034	WP	*icaA-; aap+; bhp+*	+	ERY; FA; GEN; LVX; TET; SXT;
PT13037	NP	*icaA-; aap+; bhp-*	+	FA; TET; SXT;
PT13039	WP	*icaA+; aap+; bhp-*	+	PEN; ERY; FA; LVX; MXF; TET;
PT13040	NP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; GEN; LVX; TET;
PT13041	NP	*icaA-; aap+; bhp+*	+	PEN; CLI; ERY; FA; LXV; TEC;
PT13042	NP	*icaA+; aap+; bhp-*	+	PEN; CLI; ERY; GEN; LVX; MXF; TET;

Abbreviations: NP, non-biofilm producer; WP, week biofilm producer; MP; moderate biofilm producer; SP, strong biofilm producer; -, PCR negative; +, PCR positive; nd; not detected; PEN; Penicillin; CLI, Cindamycin; ERY, Erythromycin; FA, Fusidic Acid; FOF, Fosfomycin; GEN, Gentamicin; LZD, Linezolid; LVX, Levofloxacin; RIF, Rifampin; MXF, Moxifloxacin; TET, Tetracycline; SXT, Trimethoprim/sulfamethoxazole; Underlying antibiotics demostrated an intermediary phenotype.

CHAPTER 4

RELATIONSHIP BETWEEN *ICAA*, *AAP* AND *BHP* GENES EXPRESSION AND BIOFILM DEVELOPMENT OF CLINICAL AND COMMENSAL *S. EPIDERMIDIS* ISOLATES

This chapter provides a better understanding of the interactions between biofilm-mediating *icaA*, *aap* and *bhp* genes in clinical and commensal *S. epidermidis* isolates, and their contribution to the process of biofilm formation.

4.1 Introduction

Colonization and development of biofilms on host tissues or indwelling medical devices is currently a major healthcare problem that is closely tied to the pathogenesis of this bacterium as it increases resistance to multiple classes of antibiotics [301, 302] and host immune defenses [202, 210].

The physiology of biofilm development appears to be complex and multifactorial, and bacterial colonization is considered the primary mechanism of *S. epidermidis* pathogenesis [32]. Once attached to the substrata, *S. epidermidis* cells proliferate, eventually becoming enmeshed within an extracellular polymeric substance (EPS) due to self-secretion of biomolecules, and then accumulate as multilayered cell clusters [29, 33]. A biofilm is thus defined as a structured aggregation of bacteria enclosed in a matrix consisting of a mixture of macromolecules such as polysaccharides, proteins and extracellular DNA that together protects biofilm bacteria from environmental stresses [31-33]. Poly-*N*-acetyl-glucosamine (PNAG), also designated polysaccharide intercellular adhesin (PIA), is synthesized by proteins encoded in the *icaADBC* locus [61, 62, 78] and has been for long identified as a major molecule involved in biofilm formation [69, 264]. Additionally, PIA has a significant function in the protection of *S. epidermidis* biofilm cells from host innate defenses [210] and significantly affects the tridimensional (3D) structure of mature biofilms [303]. Despite the role of PIA in biofilm formation, PIA-independent mechanisms that rely on protein-protein interactions have been described in this process [58, 135, 304]. The cell wall accumulation-associated protein (Aap) [44, 84, 305] and the homologue to the biofilm-associated protein (Bap) of *S. aureus* protein (Bhp) [86, 89-91], are the best characterized determinants involved in protein-mediated biofilm formation mechanisms. Therefore, many *in vitro* and *in vivo* studies highlighted the substantial role of PIA, Aap and Bhp molecules in the adhesion and accumulation stages of biofilm formation [45, 90, 290]. Despite their importance, molecular studies are often performed with wild-type and respective mutant strains. Thus, the impact of these genes in clinical isolates has not yet been properly studied. Moreover, it remains to be elucidated how *icaA*, *aap* and *bhp* genes may contribute to the development of biofilms by clinical isolates over time. In order to better understand the pathophysiology of *S. epidermidis*

infections, this study aims to analyze the interactions between biofilm-mediating *icaA*, *aap* and *bhp* genes in clinical and commensal *S. epidermidis* isolates, and their contribution to the process of biofilm formation, and thus, infer about *S. epidermidis* pathogenesis. For this purpose, a total of 19 *S. epidermidis* clinical and community isolates, were characterized regarding their ability to form biofilm, gene expression profile, matrix composition and their biofilm 3D structure.

4.2 Materials and Methods

S. epidermidis bacterial isolates

Twelve *S. epidermidis* clinical isolates recovered from patients with infected implanted devices (characterized in *chapter 3*) and seven commensal isolates recovered from randomly selected healthy individuals, non-workers of healthcare facilities [300], were selected from our collection. Each genetic group was defined according to the presence or absence of biofilm-mediating genes *icaA*, *bhp* and *aap* (*Table 4.1*). Three distinct *S. epidermidis* isolates (2 from the clinical setting and 1 community-acquired, whenever possible) were included per group, with the exception of those carrying a *bhp*$^+$ or *icaA*$^+$ genotype alone, which comprise only 2 isolates due to the inexistence of others carrying the same genetic trait in both collections. Additionally, all 19 isolates included in this study were previously characterized in regard to their antimicrobial resistance profile [300] and genetic similarities between those isolates were accessed by *rpoB* sequencing (*Fig. 4.1*). The DNA sequencing analysis was performed by the Eurofins MWG Operon Company *(http://www.eurofinsgenomics.eu)* using the ABI 3730XL sequencing machine.

In brief, polymerase chain reaction (PCR) was performed directly from bacterial gDNA obtained by the rapid DNA extraction method (as described in *chapter 3*) in a final volume of 60 µL. Four µL of extracted DNA were used as a template and added to 56 µL of PCR mix containing 30 µL of DyNAzyme II PCR Master Mix 2x (Finnenzymes), 4 µL of primer mixture with a concentration of 10 µM *(Table 4.2)* and 22 µL of nuclease-free water. For the detection of *rpoB* (899 bp),

the forward and reverse primers had the following sequences: 5′ – CAATTCATGGACCAAGC – 3′ and 5′ – CCGTCCCATGTCATGAAAC – 3′, respectively. The PCR reaction was performed using the MJ Mini thermal cycler (Bio-Rad) beginning with an initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 45 sec, 60 °C for 60 sec, and 72 °C for 90 sec, ending with a final extension step at 72 °C for 10 min and followed by a hold at 4 °C. Then, amplified products were analyzed in 1% agarose gel stained with Midori Green DNA stain (Nippon Genetics Europe GmbH). The PCR product was then purified using GRS PCR & Gel Band Purification Kit (GRiSP) and gDNA was quantified with a Nanodrop 1000^{TM} (Thermo Scientific), following the Eurofins MWG Operon Company requirements to sample submission. The nucleotide sequences of each isolate were analyzed using the Basic Local Alignment Search Tool *(http://blast.ncbi.nlm.nih.gov/blast.cgi)*.

Table 4.1: Distribution of the selected *S. epidermidis* isolates according to their genetic trait.

***S. epidermidis* isolates**	**Origin**	**Genotype profile** [a]
PT12006	Chapter 3	***icaA*$^{+}$*aap*$^{+}$*bhp*$^{+}$**
PT12020	Chapter 3	
SECOM030A	300	
PT11006	Chapter 3	***icaA*$^{+}$**
SECOM049A	300	
PT11003	Chapter 3	***icaA*$^{+}$*aap*$^{+}$**
PT12013	Chapter 3	
SECOM004A	300	
PT11016	Chapter 3	***icaA*$^{+}$*bhp*$^{+}$**
PT12003	Chapter 3	
SECOM020A1	300	
PT11012	Chapter 3	***aap*$^{+}$*bhp*$^{+}$**
PT12010	Chapter 3	
PT12015	Chapter 3	
SECOM040A	300	***bhp*$^{+}$**
SECOM042A	300	
PT12004	Chapter 3	***aap*$^{+}$**
PT12005	Chapter 3	
SECOM023A	300	

[a] The methods are described in *chapter 3*

Furthermore, the phenogram was constructed based on *rpoB* sequences data. The nucleotide sequences were first aligned and then the phenogram was

generated with the neighbor-joining algorithm by using the CLC Sequencer Viewer 7.6. The tree was resampled with 1000 bootstrap replications to ensure the robustness of the data.

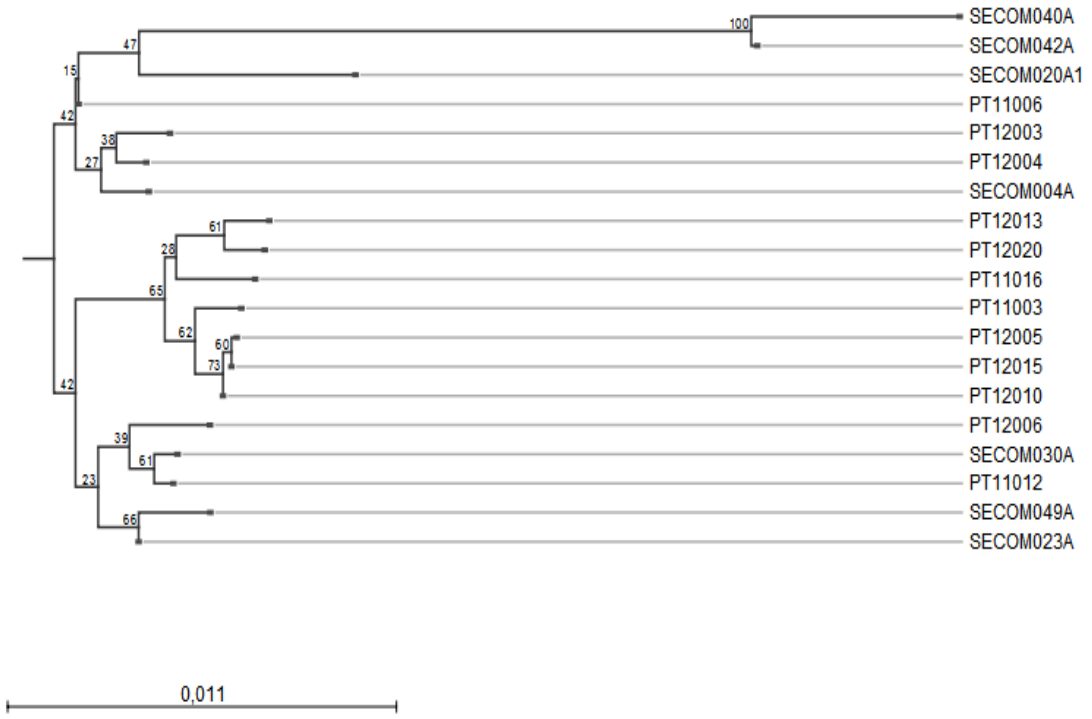

Figure 4.1: Neighbor-joining tree based on the *rpoB* gene sequences showing the phylogenetic relationships among the *S. epidermidis* isolates selected for this study. The value on each branch node is the bootstrap value (%) and the scale bar represents 0.011 changes per amino acid position.

In vitro biofilm formation

Biofilm cultures of each *S. epidermidis* isolate were performed as described in the previous *chapters* with minor modifications. Briefly, a starter culture was grown overnight in Tryptic Soy Broth (TSB, Liofilchem) at 37 °C and orbital shaking at 80 rpm (20 mm orbit diameter, Celltron, INFORS HT). Two or 10 µL of the starter culture were inoculated into 200 µL or 1 mL of TSB supplemented with 0.4% (w/v) of glucose to induce biofilm formation, in 96- or 24-well polystyrene plates (Orange Scientific), respectively. Biofilm cultures of 12, 24, 54 or 72 h, were growing in the same conditions as the starter cultures. After each 24 h, the growth medium was carefully discarded and replaced by fresh one.

Semi-quantitative biofilm assay

After 24 and 72 h of *in vitro* biofilm formation in 96-well polystyrene plates, the total biomass was assessed through a semi quantitative assay previously described and detailed in *chapter 3*. At least three independent experiments with 16 replicates each, were performed and similar results were obtained. The biofilm-forming and *icaA*-, *aap*- and *bhp*-positive *S. epidermidis* strain 9142 was used as reference strain.

Biofilm matrix disruption assay

Sodium meta-periodate ($NaIO_4$) and proteinase K which target biofilm matrix components as glucose-containing polysaccharides and proteins respectively, were tested for their ability to disrupt preformed *S. epidermidis* biofilms from polystyrene plate wells. Biofilm matrix disruption assays were performed as previously described [306, 307]. Briefly, biofilms were grown in the 96-well polystyrene plates for 72 h followed the same conditions as described above. After each biofilm formation period, the media and non-adherent cells were removed and the adherent biofilm was washed gently in 200 µL of sterile water. Then, 200 µL of 40 mM $NaIO_4$ (Sigma-Aldrich) in water or 0.1 mg/mL proteinase K (Sigma-Aldrich) in 20 mM Tris-HCl (pH 7.5) and 1 mM $CaCl_2$, were carefully added to minimize mechanical detachment of biofilms. Control wells received an equal volume of buffer without enzyme. Plates were incubated for an extra 2 h at 37 ºC, and following incubation the content of each well was discarded and washed twice with sterile water. Then, crystal violet quantification was performed as detailed in *chapter 3* to quantify the amount of stained biofilm remaining after each treatment, relative to that after treatment with the control reagent (buffer). Three independent experiments with 9 replicates for each treatment conditions were performed and similar results were obtained. *S. epidermidis* strain 9142 was used as reference strain.

Gene expression quantification

RNA extraction and cDNA synthesis: RNA extraction from 12 and 54 h-old biofilms grown on 24-well polystyrene plates, was performed following a previously

optimized protocol [308], as detailed in *chapter 2*. Briefly, the optimized protocol combines both chemical and mechanical lysis together with a column system for RNA isolation (E.Z.N.A® Total RNA kit I, Omega Bio-Tek®). After RNA extraction, gDNA was digested with DNase I (Thermo Scientific) following the manufacturer's instructions. The total RNA was quantified with a Nanodrop 1000™ (Thermo Scientific) and reverse transcribed in complementary DNA (cDNA), using the enzyme RevertAid™ H minus reverse transcriptase (Thermo Scientific) as described elsewhere [308]. The RNA integrity was assessed by agarose gel electrophoresis and visualized using a ChemiDoc™ XRS (Bio-Rad). In order to determine contamination by gDNA, a control lacking the reverse transcriptase enzyme (no-RT control) was prepared per sample. The RNA extraction and the subsequent cDNA synthesis of each biofilm with different ages of maturation per isolate were performed in triplicate.

Table 4.2: Oligonucleotide sequence used in the qPCR run.

Gene	Oligonucleotide sequence (5' to 3')	TM (°C)	PCR product size (bp)	Primer efficiency (%)
16S	FW: GGG CTA CAC ACG TGC TAC AA	59.79	176	92.5
	RV: GTA CAA GAC CCG GGA ACG TA	59.85		
icaA	Fw: TGC ACT CAA TGA GGG AAT CA	60.20	134	89.4
	Rv: TAA CTG CGC CTA ATT TTG GAT T	59.99		
aap	Fw: GCA CCA GCT GTT GTT GTA CC	59.22	190	93.9
	Rv: GCA TGC CTG CTG ATA GTT CA	59.98		
bhp	Fw: TGG ACT CGT AGC TTC GTC CT	60.01	213	95.0
	Rv: TCT GCA GAT ACC CAG ACA ACC	60.13		

Quantitative RT-PCR run: The qPCR reaction using the obtained mRNA as template was previously described [308] as detailed in *chapter 2*. The qPCR specific for *icaA*, *aap* and *bhp* were performed using iQ™ SYBR® Green supermix (Bio-Rad) with an CFX96™ Thermal cycler (Bio-Rad) setup for an initial denaturation of 10 min at 94 °C followed by 40 repeats of 5 sec at 94 °C, 10 sec at 60 °C and 15 sec at 72 °C. In addition, 16S rRNA gene that encodes the small subunit of the ribosome was used as reference gene. A mock qPCR reaction lacking the cDNA template was prepared and used as no-template control. The quantification of the mRNA transcripts, for each gene under study,

was determined using the delta Ct method (EΔCt), a variation of the Livak method [309]. Primers used in the RT-PCR are described in *table 4.2*.

Biofilm 3D structure analysis by confocal laser scan microscopy

Biofilms of selected *S. epidermidis* isolates were grown up to 72 h in 8-well Chamber Slide (Lab-Tek II; Nalge Nunc International) as described above with minor modifications. Briefly, 300 µL aliquots were added to chamber wells and incubated for 72 h at 37 ºC under agitation as mentioned above. Medium was carefully removed from wells and biofilms were rinsed with 300 µL of sterile water and stained for fluorescent confocal scanning laser microscopy (CSLM, OLYMPUS FLUOVIEW 1000) analysis. Biofilms were incubated in the dark for 15 min with 100 µL containing 0.01 mg/mL wheat germ agglutinin (WGA)-TRITC conjugate (Molecular Probes) that stains PIA molecules. Cells were stained with 100 µL of 5 µM SYTO® BC nucleic acid stain (Molecular Probes). Extracellular proteins were visualized by incubation in the dark for 30 min with 100 µL undiluted of SYPRO Ruby biofilm matrix (Molecular Probes). Stains were removed, and wells were rinsed with sterile water between each stain and before imaging. Each experiment was performed twice with technical duplicates.

Biofilm matrix composition

Extracellular biofilm matrix was collected from biofilms of selected *S. epidermidis* isolates grown in 24-well polystyrene plates for 72 h. Briefly, biofilms were scraped off, sonicated using a protocol previously optimized [310], and then centrifuged at 6000 rpm for 10 min at 4 ºC to separate biofilm cells from soluble matrix material. The soluble matrix material was then filtered using a 0.2 µm pore size filter to eliminate the intact biofilm cells and then lyophilized for further use. Proteins were determined by using the bicinchoninic acid (BCA) assay (Pierce Biotechnology) following manufactures' instructions and polysaccharides were estimated according to the procedure described by Dubois *et al.* [311], using glucose as a standard. The colorimetric experiments were performed 3 to 5 times independently per each biofilm growth period, with 3 replicates at a time. The results were expressed in µg per mL of biofilm.

Additionally, the *S. epidermidis* strain 9142 was used as a positive reference strain.

Statistical analysis

Statistical evaluations were performed with the GraphPad Prism software version 6.04 by using Student's *t*-test for the comparison of two groups. A confidence level of ≥ 95% was considered statistically significant.

4.3 Results

Biofilm formation and disruption evaluation

Based on the previously established criteria, 19 isolates from different origins and showing different phylogenetic relationships (*Fig. 4.1*) were selected in order to assess the genetic contribution of *icaA*, *aap* and *bhp* in biofilm formation by clinical and commensal isolates of *S. epidermidis*. For this purpose, all strains were incubated up to 72 h and *in vitro* biofilm-formation was assessed.

As illustrated in *figure 4.2*, the isolates carrying the *icaA* gene were more prone to form larger amounts of biofilm and this was more evident at older biofilms (72 h-old). In fact, all isolates, with the exception of PT12015, had increased their biofilm biomass over time and some (31.6%, n = 6) were as strong biofilm producers as the reference strain 9142. No differences were found in the degree of biofilm formation between isolates that carry *icaA* alone or in combination with *aap* and/or *bhp.* Furthermore, *S. epidermidis* isolates that do not carry the *ica* operon displayed less capacity to form biofilm when compared to *icaA*$^{+}$ isolates.

A few studies have referred that the proportion of polysaccharides and proteins in the biofilm matrix greatly varies among different strains and it is dependent on the growth conditions [312]. In order to better understand those differences and the nature of the molecules involved in the biofilm structure, we have tested the sensitivity of 72 h-old biofilms to $NaIO_4$ and proteinase K – two biofilm degrading agents (*Fig. 4.3*). Polysaccharides undergo oxidation by $NaIO_4$ [313], while proteins were targeted and digested by proteinase K [314].

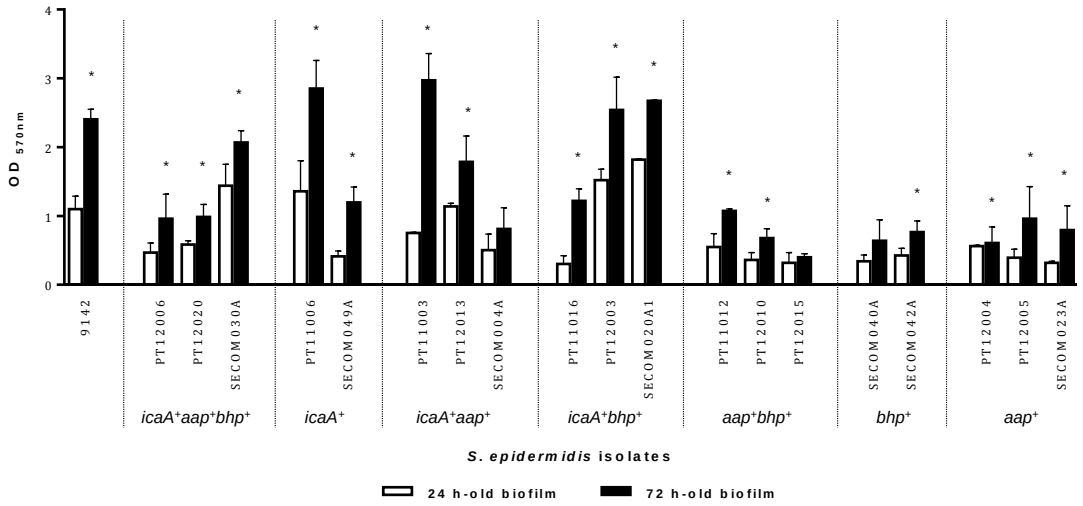

Figure 4.2: Characterization of biofilm formation by *S. epidermidis* clinical and commensal isolates during 24 and 72 h of growth. Bars correspond to average values of measurements taken from 16 duplicate wells of at least 3 independent assays and error bars represent the standard deviations. Statistically significant differences observed between biofilms grown for 24 and 72 h are indicated by the asterisks (*). The *S. epidermidis* 9142 was used as a reference strain.

As expected, preformed biofilms of strain 9142 were efficiently dispersed by $NaIO_4$ while it remained nearly intact after proteinase K digestion [315]. A similar effect was found on *icaA*$^+$*aap*$^+$*bhp*$^+$ biofilms. Not surprising, biofilms from the isolates belonging to the *aap*$^+$ group were partially digested by proteinase K although not sensitive to the action of $NaIO_4$. Furthermore, similar effect was expected in *bhp*$^+$ and *aap*$^+$*bhp*$^+$ groups.

Regarding *icaA*$^+$ *S. epidermidis* isolates, we have found overall an increased effect of $NaIO_4$ in biofilm disruption, as expected. In addition, *S. epidermidis* strains with higher sensitivity to $NaIO_4$ were those showing thicker biofilms by 72 h of growth. There were, however, some exceptions. For instance, despite the presence of *icaA* gene, $NaIO_4$ had little effect on the disruption of the biofilm of SECOM049A (*icaA*$^+$), SECOM004A (*icaA*$^+$*aap*$^+$) and PT11016 (*icaA*$^+$*bhp*$^+$) isolates. Interestingly those strains did not form thick biofilms suggesting a lower content in polysaccharides.

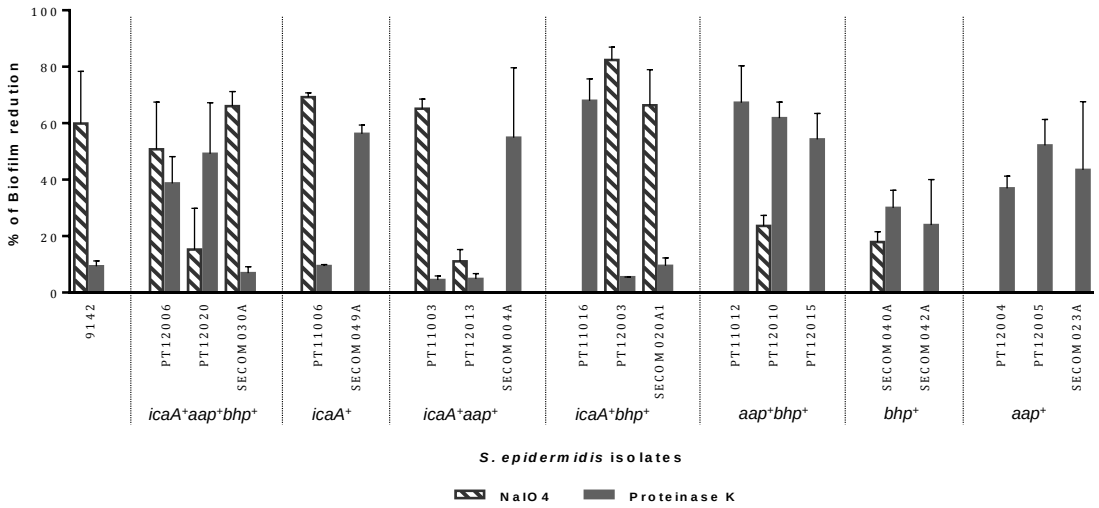

Figure 4.3: Effects of $NaIO_4$ and proteinase K on pre-formed 72 h-old biofilms of *S. epidermidis* clinical and commensal isolates. Bars are the mean averages of 3 independent experiments with 9 replicates and the error bars represent the standard deviations. The percentage of biofilm reduction was calculated according to the following formula: 1 - (Abs $_{\text{buffer plus enzyme}}$ / Abs $_{\text{buffer alone}}$) x 100. The *S. epidermidis* 9142 was used as a reference strain.

The effect of *icaA,* aap and *bhp* expression in *S. epidermidis* isolates biofilm formation

While many studies have related biofilm formation to the presence of known biofilm-associated genes, fewer studies have studied the expression of those genes and its consequence in biofilm accumulation. In order to assess the relative contributions of *icaA*, *aap* and *bhp* expression in biofilm accumulation, RNA was extracted in earlier (12 h) and later (54 h) stages of biofilm formation and specific gene expression was assessed by qPCR. The expression levels of *icaA*, *aap* and *bhp* biofilm-mediating genes are presented in *table 4.3*. By normalizing gene expression to that of reference 16S rRNA gene, we were able to quantify the relative expression of each assessed gene. An interesting overall observation was that within each isolate, we found gene expression variability up to 500 fold (*bhp* was expressed 499 fold higher than *aap*, in PT12015 at 54 h), independently of the condition tested. Also independently of the genetic background, isolates with higher biomass and with a higher increase in biomass over time were the ones with the highest increase in *icaA* expression from 12 to 54 h of growth. Such an example is observed in the ~23-fold increase in *icaA* expression (6.76±2.52E-05 at 12 h *versus* 1.58±0.39E-03 at 54 h) observed in

Table 4.3: Gene expression analysis in 12 h- and 54 h-old biofilms of clinical and commensal isolates of *S. epidermidis.*

Gene status group	*S. epidermidis* isolates	mRNA expression normalized to 16S RNA								
		bhp			*aap*			*icaA*		
		12 h	54 h	*p*-value*	12 h	54 h	*p*-value*	12 h	54 h	*p*-value*
***icaA*⁺*aap*⁺*bhp*⁺**	**PT12006**	1.61±0.56E-05	2.52±0.91E-05	0.002	1.35±0.43E-04	1.71±0.30E-04	0.047	2.12±1,22E-06	1.01±0.86E-05	0.003
	PT12020	2.34±1.13E-05	2.61±1.53E-05	> 0.05	1.27±0,50E-04	1,55±0.94E-04	> 0.05	2.75±2,11E-06	1.20±0.44E-06	> 0.05
	SECOM030A	2.98±1.73E-05	1.95±0.78E-05	> 0.05	1.72±0.51E-04	4.31±1.12E-04	< 0.0001	3.17±1.56E-05	1.50±0.80E-04	0.002
***icaA*⁺**	**PT11006**							3.47±1.86E-05	6.91±2.46E-05	0.013
	SECOM049A							1.75±0.80E-05	3.00±0.87E-05	0.002
***icaA*⁺*aap*⁺**	**PT11003**				1.89±0.81E-05	7,05±0,60E-05	< 0.0001	6.76±2.52E-05	1.58±0.39E-03	< 0.0001
	PT12013				3.00±1.33E-05	7,67±5,76E-05	0.044	2.83±0.13E-05	5.16±0.61E-05	< 0.0001
	SECOM004A				1.77±1.29E-05	2.75±1.65E-05	0.006	6.07±5.68E-07	1.28±1.23E-06	> 0.05
***icaA*⁺*bhp*⁺**	**PT11016**	1.02±0.86E-06	1.30±1.13E-05	0.003				9.50±0.10E-08	3.57±2.44E-07	0.011
	PT12003	2.82±1.39E-06	6.51±2.58E-06	0.001				1,08±0.54E-04	3.93±2.56E-04	0.002
	SECOM020A1	3.20±1.61E-05	5.07±4.23E-05	> 0.05				4.70±2.32E-05	1.98±2.55E-04	0.045
***aap*⁺*bhp*⁺**	**PT11012**	2.22±0.63E-06	2.82±1.71E-05	0.002	9.14±2.28E-06	5.01±3.74E-05	0.003			
	PT12010	4.78±2.81E-06	2.87±1.08E-05	0.001	3.68±1.72E-07	1.69±1.08E-05	0.011			
	PT12015	3.61±1.40E-06	1.02±0.11E-04	< 0,0001	2.18±1.06E-07	2.05±1.30E-07	> 0.05			
***bhp*⁺**	**SECOM040A**	6.03±0.67E-06	1.49±0.56E-05	0.003						
	SECOM042A	1.02±0.86E-06	1.78±0.95E-06	> 0.05						
***aap*⁺**	**PT12004**				3.84±2.78E-05	2.15±0.84E-04	< 0.0001			
	PT12005				1.78±0.93E-05	2.76±1.17E-05	0.048			
	SECOM023A				2.56±1.43E-05	3.08±0.60E-05	> 0.05			

*$p < 0.05$ by Student's *t*-test was used for the comparison of the levels of gene expression between 12 and 54 h-old biofilms.

PT11003 isolate and the respective increase in biofilm observed. Amongst these isolates, PT12015 showed the less extent of biofilm formation and this associated with a significantly lower expression of *aap*, especially at 54 h. Interestingly, a ~28-fold increase in *bhp* expression between 12 and 54 h was observed, but no increase in biomass was detected.

Biofilm 3D structure and matrix composition

Based on the previous results, we became interested in further exploring the phenotype of a few isolates. For this purpose, PT11006 (*icaA*$^+$), PT12004 (*aap*$^+$), SECOM040A (*bhp*$^+$) and PT11003, PT12013 (*icaA*$^+$*aap*$^+$) and, PT12003 and SECOM020A1 (*icaA*$^+$*bhp*$^+$) isolates were visualized by CLSM *(Fig. 4.4)* and the amount of proteins and polysaccharides was quantified.

Confirming our previous gene expression determinations, biofilm accumulation and detachment data, CLSM imaging confirmed that SECOM040A (*bhp*$^+$) and PT12004 (*aap*$^+$) exhibit a flat 3D biofilm structure, harboring lower biomass than PT11006 (*icaA*$^+$). Conversely, the *icaA*$^+$ isolate exhibited the typical “mushroom” structure which is highly associated with *ica*-mediated biofilm growth. Furthermore, we detected 19±4 μg of proteins in the matrix per mL of biofilm in PT11006 clinical isolate, while both SECOM040A and PT12004 only had 11±3 μg/mL and 13±3 μg/mL, respectively. This is not surprising since the total biomass of PT11006 was higher comparatively to *icaA*$^-$ isolates, and many more proteins can be found in the matrix than Aap or Bhp. The analysis of the *icaA*$^+$*aap*$^+$ group also confirmed our previous observations, having PT11003 a higher cell density and polysaccharide-content than PT12013. The higher concentration of polysaccharides was also confirmed by the Dubois quantification (28±11 μg/mL in PT11003 *versus* 5±2 μg/mL in PT12013). Interestingly, the concentration of proteins in the matrix was more similar (16±4 μg/mL in PT11003 *versus* 19±7 μg/mL in PT12013). Despite the different origins, the PT12003 clinical isolate and SECOM020A1 commensal isolate – both belonging to the *icaA*$^+$*bhp*$^+$ group, exhibited a very similar biofilm structure. Nevertheless, while the polysaccharide concentration was similar in both biofilms (7±3 and 7±4 μg/mL, respectively), PT12003 had a significant higher protein content in the matrix (56±7 μg/mL *versus* 19±8 μg/mL). Interestingly,

overall CLSM analysis revealed that polysaccharides usually accumulate in clusters while proteins appear to be more evenly distributed through the biofilm.

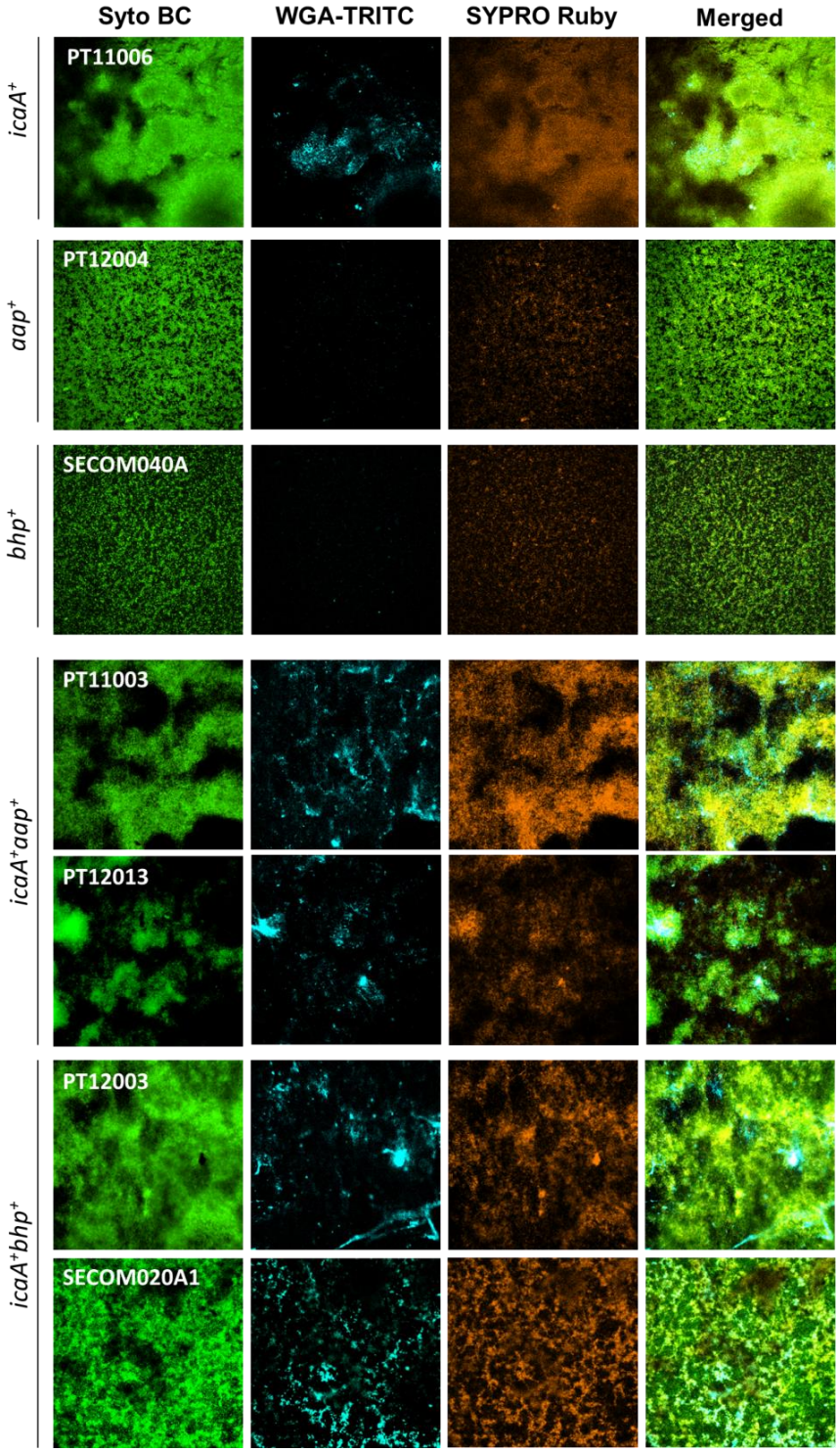

Figure 4.4: CLSM observation of 72 h-old biofilms formed by isolates of *S. epidermidis.* Triple staining was done with SYTO® BC for nucleic acids, WGA-TRITC that stains the extracellular PIA and with SYPRO® Ruby that stain the proteinaceous content. The last picture is the merged image of the three channels. Magnification of ×400.

4.4 Discussion

Understanding how specific virulence-associated genes individually influence the biofilm accumulation and proliferation in clinical and/or commensal isolates, is of upmost importance. Independent reports have shown that biofilm formation by *S. epidermidis* is influenced by different molecular mechanisms. Furthermore, *in vitro* quantification of the biofilm proliferation, the transcriptional analysis of the genes that mediate this biofilm formation and the estimation of the biochemical composition of the biofilm matrix of *S. epidermidis* isolates, will improve the understanding of the phenotypic variations that might led to the *in vivo* adaption of *S. epidermidis* isolates. With this in mind, we selected 19 *S. epidermidis* isolates according to their genetic trait and then studied the relationship between *icaA*, *aap* and *bhp* transcription and the biofilm phenotype exhibited by clinical and commensal isolates grown over time and at the same *in vitro* conditions.

Overall our results have shown that *icaA*$^{+}$ isolates were unquestionably the stronger biofilm producers, independently of the time of growth tested (up to 72 h). Nevertheless, it is still important to understand how this growth *ie*, biofilm development, is related to the expression of specific genes and to the composition of extracellular matrix. As biofilm regulation is a cascade of cross-linked events working together to maintain the biofilm structure, it is conceivable that bacterial isolates with a different genetic pattern could display similar phenotypes. In order to infer about the biochemical composition of the biofilm matrix of each individual isolate, we have investigated the susceptibility of pre-formed 72 h aged biofilms to two biofilm-degrading agents ($NaIO_4$ and proteinase K). As expected, proteinaceous biofilms were digested by proteinase K while *S. epidermidis ica*-dependent biofilm were highly sensitive to $NaIO_4$ therefore demonstrated the importance of polysaccharides in those biofilms.

Aiming at further understanding the impact of biofilm-related gene expression on the phenotypic variations observed between *S. epidermidis* isolates, we had quantified the *in vitro* expression of *icaA*, *aap* and *bhp* genes in 12 h- and 54 h-old biofilms. Ours results showed that the isolates with higher *icaA* expression also formed larger amounts of biofilm which was not surprising, since most well described *S. epidermidis* strains considered stronger biofilm producers, carry the *icaADBC* operon. While *icaA* gene expression was more pronounced at 54 h (with

the exception of the PT12020 isolate) *aap* was the most expressed gene at earlier and later stages of biofilm growth within *icaA*$^{+}$*aap*$^{+}$*bhp*$^{+}$ group. Interestingly, the higher *aap* expression do not correlated to a higher biofilm formation. However, it has been suggested that, during *in vitro* biofilm accumulation assays, the presence of PIA may significantly mask the contribution of Aap [88] which can in part justify the lower biofilm production and proliferation of PT12006 and PT12020 isolates. Together, those findings pointed out that the expression of the *icaA* gene is still the major contributor to the biofilm growth at larger-scales in *in vitro* assays. Interestingly, a dose-dependent effect on *bhp* expression and subsequent biofilm accumulation was not detected, while in *aap* a variable effect was found. Hennig and colleagues [82] reported that in contrast to *bhp* expression level, the expression of *aap* was upregulated in an *icaC*::IS256 but biofilm-positive mutant, and thus considered responsible for the biofilm formation. In fact, this finding might explain the biofilm phenotype observed in the PT12015 isolate (*aap*$^{+}$*bhp*$^{+}$). Despite the 28-fold increase in the expression of *bhp*, this clinical isolate demonstrated a lesser extent in biofilm proliferation which could be therefore associated to the insignificant lower increase in expression of *aap* at 54 h (less that 1-fold). Moreover, the clinical PT11016 (*icaA*$^{+}$*bhp*$^{+}$) and the commensal SECOM004A (*icaA*$^{+}$*aap*$^{+}$) and SECOM049A (*icaA*$^{+}$) isolates reveal lower levels of *icaA* transcription and biofilm biomass when compared to the other members of the group. In addition, $NaIO_4$ had no or little effect on biofilm disruption, further suggesting a minor role of polysaccharides in the biofilm formation by these isolates. As PT11016 was recovered from a patient with an infected implanted device, it remains a possibility that more pronounced levels of *icaA* expression may occur *in vivo* than in the used *in vitro* conditions. Additionally, the levels of *icaA* expression could also be modulated by *icaADBC* inactivation either due to the insertion/excision of IS256 or IS257 elements in *icaADBC* genes [316, 317] or by the action of the *icaR* repressor [70]. Similar results were therefore observed by Chokr *et. al.* [81] and Cafiso *et. al*. [318]. Besides genetic modifications, several studies had established that the expression of *icaADBC* appears to be highly variable among clinical isolates [72, 319, 320] and the biofilm formation is a result of several stimuli [81, 321].

By CLSM analysis we could observe that the structures of the biofilm matrix of *icaA*$^{+}$ and *icaA*$^{-}$ are quite distinct. The structural morphology of the matrix formed

by protein-dependent biofilms is indeed less complex than in PIA-dependent biofilms, as reported before [290, 322]. Through this work, no phenotypic or genotypic differences were observed among clinical and commensal *S. epidermidis* isolates, suggesting that both had similar capacity to develop biofilms *in vitro* overtime, as previously reported by others [295, 323-325]. This observation can be supported by the analysis of the phylogenic tree as it provides some indication of the phylogenetic relationships between the studied clinical and commensal isolates. As observed, some isolates with different origins appears to be closely related although phenotypically and genotypically distinct from each other (*e.g.* PT12003, PT12004 and SECOM004A). Additionally, both types of isolates demonstrated similar biofilm structure by CLSM.

Collectively, our results showed that *icaA* gene is highly correlated to the biofilm lifestyle and revealed that the highly complex biofilm structure (bacteria and matrix) is much more diversified among *S. epidermidis* clinical isolates than previously described in the so called, “reference” strains. While we’ve confirmed that *aap* plays an important role in PIA-independent biofilms, only *S. epidermidis* isolates harboring the *ica* gene demonstrated the ability to develop thicker biofilms with a more complex 3D biofilm organization. Importantly, we were not able to significantly associate *bhp* expression to biofilm formation, in opposition to studies performed in *S. aureus* strains [89, 326]. Nevertheless, other studies point out that isolates of *S. epidermidis* do not necessarily have the same response to *in vitro* and *in vivo* conditions [327] and therefore further *in vivo* research should be conducted on those isolates.

CHAPTER 5

FINAL NOTES AND FUTURE PERSPECTIVES

This chapter concludes this thesis and suggests future work.

5.1 Concluding remarks

The incidence of HAIs caused by *S. epidermidis* has been on the rise due to the increased use of medical implant devices [328, 329]. The pathogenesis of these infections relies on bacteria adherence to the biomaterial and subsequent accumulation, and production of large amounts of polysaccharides, proteins and other extracellular substances [33, 330]. The bacteria within the formed biofilm structure are protected against the host phagocytes [202, 206] and are less effectively cleared by antibiotic treatment, if at all [278, 331]. Thereby, removal of infected implants is a necessary solution, which highly increases the costs of medical care [6, 328]. Since it is highly likely that implants will be increasingly used as a means to treat or manage a variety of medical conditions, it is therefore essential to develop improved strategies that could limit the risk of colonization of those implants and to effectively combat infections when they occur. The development of preventive strategies will, in part, require a more complete understanding of bacterial factors involved in the biofilm lifecycle. In this context, this thesis was focused on the study of *S. epidermidis* biofilm cell accumulation and maturation with a particular emphasis on the understanding of how specific biofilm-mediating genes contribute to those stages of *S. epidermidis* biofilm development.

Throughout this thesis, the genotype-to-phenotype relationship of Portuguese *S. epidermidis* isolates was addressed, with particular attention to the *ica operon*, *aap* and *bhp* - three main biofilm-related genes involved in biofilm formation. *Chapter 2* described the troubleshooting performed with two key techniques used to quantify biofilm cells throughout this work. Although widely used, both techniques were not yet properly optimized to use with older *S. epidermidis* biofilms, composed of highly aggregated cells. The achievements described in *chapter 2* contributed to a more accurate quantification of biofilms at older development stages. In *chapter 3*, a local epidemiologic study was described which included the phenotypic and molecular features of 86 Portuguese *S. epidermidis* clinical isolates and their association to patient-reported outcomes. To the best of our knowledge this was the first study addressing the problematic of *S. epidermidis* biofilm-related infections in Portugal. Although limited by the number of used isolates, our data provided evidence that high rates of antimicrobial tolerance are indeed related to higher consumption of antibiotics inside healthcare institutions. The results

presented in *chapter 4* describe the main objective of this thesis, *i.e*. to understand how *ica*, *aap* or *bhp* may contribute to biofilm maturation in bacterial isolates. A selection of previously characterized commensal isolates was also included in this study, in order to determine if the reported findings in clinical isolates were also valid for commensal isolates. Ours findings highlighted that *icaA* was the most important factor contributing to the formation of strong biofilms by *S. epidermidis* isolates. Additionally, no differences were found between clinical and commensal isolates, a result that reinforces the concept that *S. epidermidis* is an accidental pathogen [28], suggesting that *S. epidermidis* isolates found as part of the normal skin flora have the potential to cause disease.

5.2 Future perspectives

The results presented throughout the different chapters of this thesis, provided further insights on the *S. epidermidis* fitness while exploring the contributions of *icaA*, *aap* or *bhp* biofilm-mediating genes in biofilm accumulation and maturation.

A major limitation of these findings is related to the fact that, biofilm growth was characterized using conventional *in vitro* approaches. However, to effectively treat and prevent biofilm-related infections, it is a priority to understand how bacterial biofilms are established and progress within the human body. In fact, it remains debatable whether *in vitro*-formed biofilms actually resemble biofilms formed *in vivo* eventually causing of *S. epidermidis* persistent and recalcitrant infections. In line with this statement, future research should be performed with *in vitro* flow cell systems or in representative animal models (such as, mice [332, 333], rabbits [334] or guinea pigs [335]), to more accurately simulate *in vivo* human conditions. Flow cell chambers would be a useful tool for an increased knowledge about the biofilm dynamics within the human body, while animal models would better mimic the dynamic interaction between host-, bacterial-, and environmental factors during *S. epidermidis* colonization. However, *in vivo* assays are quite expensive and limited by ethical issues implying that experimental works need to be strategically conducted. Therefore, an important step, previous to *in vivo* studies, could be the inclusion of *ex vivo* approaches that mainly consist in improved *in vitro* assays where the liquid broth is replaced with human blood [336, 337].

Finally, all models associated to high-throughput “omics” technologies would provide a global picture of gene expression and protein synthesis, and thus a more comprehensive understanding of the pathogenesis of *S. epidermidis* and of its behavior within the human body, which is in fact, the ultimate goal.

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